The effect of the endocannabinoid anandamide on human endometrial stromal cells

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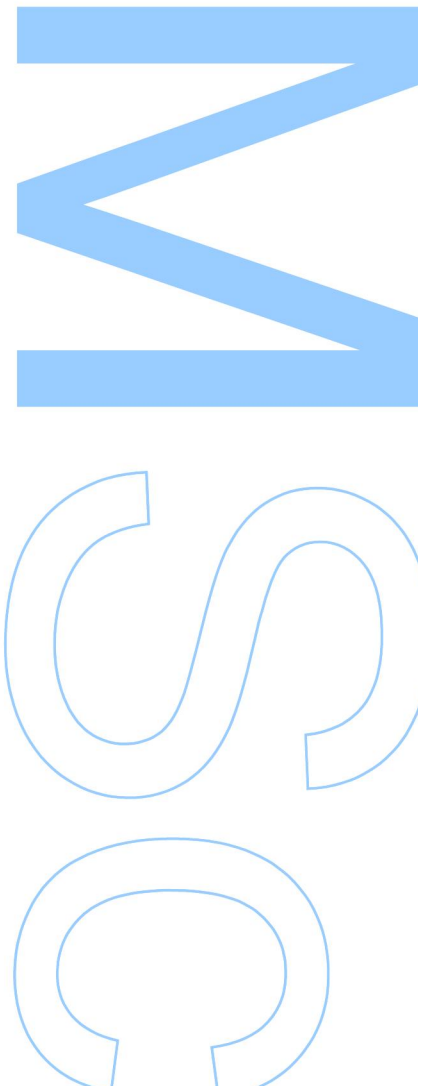

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Abstract

Each month, the endometrium undergoes a cyclic process of proliferation and differentiation establishing the basis for a successful pregnancy. Due to a tight modulation between gonadotrophins, pituitary and ovarian hormones, the endometrium only becomes receptive in a specific “window of implantation”. During this time, endometrial stromal cells differentiate into decidual cells, which control trophoblast invasion, regulate immune responses and restrain the oxidative stress. Nowadays, there is an emergent research area that links the endocannabinoid system with key events for a successful pregnancy. The main endocannabinoid, anandamide (AEA), has been described to be involved in folliculogenesis, embryo transport, decidualization and placental development. Although it is known that endocannabinoids are involved in rat decidual remodelling, it is not clear the impact of these lipids mediators on human endometrial stromal cell proliferation and differentiation. Thus, this work aims to fill this gap in scientific knowledge by studying the morphological and biochemical effects of AEA on human endometrial stromal cells. AEA induced an apoptotic and anti-proliferative effect on non-differentiated cells. On the other hand, AEA also led to the presence of binucleate cells and prevented the process of differentiation. Altogether, this suggests that AEA may be enrolled in the regulation of endometrial remodelling, and thus, interfere with pregnancy establishment.

Resumo

O endométrio sofre um processo cíclico de proliferação e diferenciação constituindo a base para uma gravidez de sucesso. A modulação entre as hormonas do hipotálamo, hipófise e ovário determinam a recetividade do endométrio durante um período específico denominado por "janela de implantação". As células do estroma do endométrio diferenciam-se em células deciduais, que controlam a invasão dos trofoblastos, a resposta imunitária e a defesa contra o stress oxidativo. A investigação do sistema endocanabinóide emergiu nos últimos anos e relaciona este com os processos essenciais para uma gravidez bem sucedida. O principal endocanabinóide, a anandamida (AEA), parece estar envolvido na maturação folicular, no transporte do embrião, na decidualização e no desenvolvimento da placenta. Embora os endocanabinóides estejam envolvidos na remodelação decidual do rato, ainda não é claro o impacto destes mediadores lipídicos na proliferação e diferenciação das células do endométrio humano. Assim, este trabalho tem como objetivo colmatar esta falha no conhecimento científico através do estudo dos efeitos morfológicos e bioquímicos da AEA nas células do estroma uterino. A AEA induziu um efeito apoptótico e anti-proliferativo nas células endometriais não diferenciadas. Por outro lado, a AEA também levou ao aparecimento de células binucleadas e preveniu o processo de diferenciação. Estes resultados sugerem que a AEA poderá participar na regulação da remodelação endometrial e por conseguinte interferir com o desenvolvimento de uma gravidez de sucesso.

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2-AG	2-arachidonoylglycerol
2-AGE	2-arachidonoylglyceryl ether
AA	Arachidonic acid
ABHD	α-β-hydrolase domain
AC	Adenylyl cyclase
AEA	Arachidonoylethanolamide
AIBPs	AEA intracellular binding proteins
AMT	Anandamide membrane transporter
ART	Assisted reproduction technologies
C/EBPb	CCAAT/ enhancer-binding protein
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
COX-2	Cyclooxygenase-2
DAG	1,2-diacylglycerol
DAGL	Diacylglycerol lipase
DSC	Decidualized stromal cells
E_2	17-β-estradiol
ECM	Extracellular matrix
ECS	Endocannabinoid system
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
$EtNH_2$	Ethanolamine
FAAH	Fatty acid amide hydrolase
FABP	Fatty-acid-binding proteins
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FLAT	FAAH-like anandamide transporter protein
FSC	Forward light scatter
FSH	Follicule stimulating hormone
Fsk	Forskolin
FOXO1	Forkhead box protein O1
GnRH	Gonadotropin-releasing hormone
GnSAF	Surge-attenuating factor
GPCRs	G-protein-coupled receptors

HPG	Hypothalamic-pituitary-gonadal
hTERT	Human telomerase reverse transcriptase
IGFBP1	Insulin-like growth factor binding protein
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LOX	Lipooxigenase isoenzymes
MAG	Monoacylglycerols
MAGL	Monoacylglycerol lipase
MM1	Minimal medium 1
MMP	Matrix metalloproteinases
MPA	Medroxyprogesterone acetate
MTT	3-(4,5-di-methylthiazol-2-yl)-2,5-difenyltetrazolium
NAAA	N-acylethanolamine-hydrolyzing acid amidase
NADA	N-arachidonoyldopamine
NAE	N-acylethanolamines
NAGly	N-arachidonoylglycine
NAPE	N-arachidonoyl-phosphatidylethanolamine
NAPE-PLD	Phospholipase D
NAT	N-acyltransferase
NK	Natural killer
PBS	Phosphate buffered saline
PC	1-arachidonoyl-phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3'-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phopholipase C
PPARs	Peroxisome proliferator-activated receptors
PR	Progesterone receptor
PRL	Prolactin
RLU	Relative light units
ROS	Reactive oxygen species

RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SM	Sphingomyelin
SMase	Sphingomyelinase
SPT	Serine palmitoyltransferase
SSC	Side light scatter
STS	Staurosporine
STAT5	Signal transducers and activators of transcription 5
THC	Delta-9-tetrahydrocannabinol
TRP	Trasient receptor potential
uNK	Uterine natural killer
VEGF	Vascular endothelial growth factor
ODA	Cis-9,10-octadecanoamide

I Introduction

1 Endometrium

The endometrium is the mucosa that lines the lumen of the uterus. It is composed of two distinct layers. The luminal functional layer (***stratum functionalis***) contains glands lined with columnar epithelium that extend from the surface epithelium and a loose stroma, while the basal layer (***stratum basalis***) adjacent to the myometrium comprises the bases of the endometrial glands surrounded by dense stroma (1). In every menstrual cycle, the upper functional layer proliferates, secretes and sheds, while the lower basal layer is permanent and provides cells for generating a new functional layer (2).

During the reproductive phase, the endometrium becomes a highly dynamic structure regulated by the ovarian hormones, progesterone and estradiol. Human endometrium undergoes precise defined morphological changes with the aim of creating a suitable environment in the right window of time (from day 21 to day 24 of menstrual cycle) to receive the embryo. The cyclic control between gonadotrophins and ovarian steroids, is the key for a receptive endometrium and subsequently for a successful implantation.

1.1 The menstrual cycle

Every month, during reproductive lifespan, the women's body undergoes a series of changes that leads to ovulation and prepares the uterus to receive the fertilized oocyte. When conception does not occur, the superficial endometrial layer starts to shed and the menstrual cycle is reinitiated. Menstrual cycle length is highly variable, ranging from 25 to 34 days in young women and also presents lifelong variations from menarche to menopause (3).

The human menstrual cycle is characterized by a highly coordinated interplay between ovarian, pituitary and hypothalamic hormones, which are regulated by positive and negative feedback mechanisms. Before puberty, the hypothalamus is extremely sensitive to the suppressing effect of the very low levels of circulating estrogen. However, as puberty approaches, the hypothalamus becomes less sensitive resulting in the derepression of the gonadostat and a subsequent increasing secretion of gonadotrophins (4). From the time of puberty onward, the hypothalamic neurones, under the control of diverse neurotransmitters, secrete gonadotrophin-releasing

hormone (GnRH) in a pulsatile manner, which stimulates the production and secretion of follicule stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary gland. This stimulates the secretion of ovarian estrogen (estradiol) and progesterone, which in turn modulates the relative amounts of LH and FSH at the pituitary and regulates GnRH at the hypothalamus (5,6).This highly regulated chain of neuro-endocrine phenomena is responsible for the ovary and uterus extensive remodelling throughout follicular/proliferative and luteal/secretory phases (Figure 1).

1.1.1 Follicular or proliferative phase

Until the 14th day of the menstrual cycle the growing ovarian follicles produce estrogen, which are responsible for the proliferation of the endometrium. The human ovary contains follicles at different stages of development, from the primordial to the preovulatory size. Although the initial recruitment of follicles is not dependent on gonadotropins, these hormones play a crucial role in their maturation and FSH, in particular, is responsible for the selection of the dominant follicle (7). During the process of maturation, follicles develop two layers of steroidogenic tissues constituted by granulosa cells surrounded by theca cells, which are responsible for the synthesis of ovarian steroidal hormones (8). In women, the action of these hormones on gonadotropin secretion can be investigated by administrating exogenous steroids or selective estrogen receptor modulators, by eliminating endogenous hormones through ovariectomy or by enhancing endogenous estrogen activity through ovarian stimulation (4).

Although estradiol is the main secretory product of the follicle, progesterone is also produced in lower concentration by the ovaries during follicular phase (9). Both these ovarian steroids have an important role in the control of gonadotrophins secretion, in the context of the negative feedback mechanism (10), though the secretion of FSH does not only depend on estradiol and progesterone (11). Apart from the steroids, the ovaries also produce non-steroidal substances, such as inhibins (10). The major form of inhibin secreted during follicular phase is inhibin B. Its serum levels rise sharply from early follicular phase and declines thereafter until midcycle, while during luteal phase the concentration is low (10,12).

In the follicular-luteal transition, there is a characteristic LH/FSH surge, due to the activation of a positive feedback mechanism via the secretion of high amounts of estradiol by the ovulatory follicle. This steroid sensitizes the pituitary to the hypothalamic GnRH (13), which together with the increasing concentration of progesterone and the reduced bioactivity of gonatrophin surge-attenuating factor

(GnSAF) is responsible for the modulation of the surge (14,15). The gonadotrophin surge is the physiological trigger to initiate a cascade of proteolytic events that control ovulation. Via its receptor, LH activates adenylyl cyclase and stimulates the protein kinase A (PKA) pathway, induces the rapid and transient expression of specific genes critical for ovulation in granulosa cells of preovulatory follicles (16). LH surge selectively induces the biosynthesis of cyclooxygenase-2 (COX-2) in granulosa cells, which in turn produces a rapid rise in follicular fluid prostaglandin E_2 (PGE_2) (17). The synthesis of biologically active prostaglandins by follicular cells seems to be an important factor for follicular rupture (18). However, their mechanism of action still remains to be elucidated (17).

1.1.2 Luteal or secretory phase

During the secretory phase, progesterone inhibits endometrial growth and the endometrium undergoes morphological and biochemical changes through a process called decidualization. This reaction is characterized by modification of uterine glands, influx of specialized natural killer cells, vascular remodelling and transformation of endometrial stromal fibroblasts into decidual cells.

In the ovary, besides the activation of the machinery that leads to ovulation, the LH surge is also responsible for promoting the differentiation of somatic cells into luteal cells (19). The primary function of the corpus luteum is the secretion of progesterone, although it still retains the ability to produce some estradiol (20). These steroids control gonadotrophin secretion during luteal phase by a negative feedback mechanism. In contrast to inhibin B, inhibin A concentration is low in the follicular phase but increases to its maximal during the midluteal phase (21). Inhibin A has been shown to mediate negatively the FSH secretion (22), becoming one of the important components of the negative feedback control of gonadotrophin during this phase.

During the passage from the luteal to the next follicular phase, an increase in serum FSH concentration occurs typically 2-3 days before the onset of the menstrual period (23). This intercycle rise of FSH is the result of the significant decline in estradiol and progesterone as well as inhibin A concentrations toward the end of the luteal phase and is responsible for the selection of the dominant follicle of the next cycle (10).

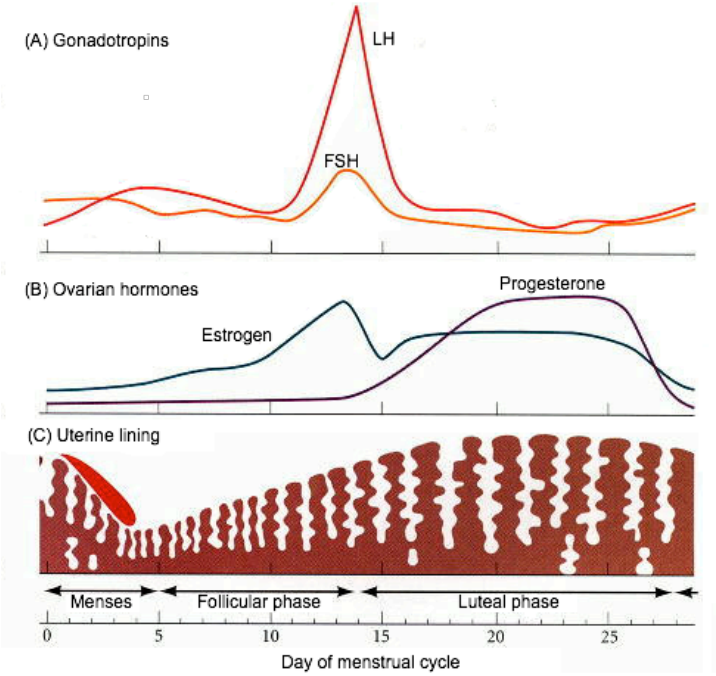

Figure 1. The human menstrual cycle. Coordination between gonadotropins (A) and ovarian hormones (B) is essential to regulate cyclic endometrial modifications (C). Adapted from (24).

1.2 Endometrial remodelling

Cyclical growth and decline of the endometrium is universal in non-pregnant mammals, but external loss of blood is only seen in primates and in very few other species (24). The process of menstruation occurs after progesterone withdrawal at the end of each menstrual cycle and is characterized by the loss of most of the *stratum functionalis* accompanied by bleeding (25). Menstruation has many of the features of an inflammatory process. Progesterone has anti-inflammatory properties, and its rapid declining levels (along with estradiol) in the late secretory phase initiates a sequence of interdependent events of an inflammatory nature. Progesterone withdrawal induces the release of NF-κB from IκB leading to inflammatory gene expression, resulting in an influx of inflammatory cells. Interactions between these and decidualized stromal cells mediate the release of a wide variety of pro-inflammatory mediators including chemokines, cytokines and prostaglandins (26). The resultant leukocyte recruitment mediates the activation of matrix metalloproteinases (MMP), which is a prerequisite for tissue degradation at menstruation (26,27).

Although it was initially thought that the endometrial repair was a subsequent process of menses, recent studies have suggested that shedding and repair occur simultaneously in adjacent areas during menses (28). This process of regeneration

shares common features with events of classic tissue injury and repair. Wound healing is a dynamic interactive process involving soluble mediators, blood cells, extracellular matrix and parenchymal cells. Similar to endometrial repair, it involves temporally overlapping phases of inflammation, tissue formation, remodelling and angiogenesis (1,25). The inflammatory process that is the basis of menstruation is also the initial point for the repair process. The occurrence of the apoptotic process is crucial for the resolution of inflammation, in particular by limiting the presence of inflammatory cells (1).

The first stage of endometrial regeneration is the re-epithelialization, after which occurs the rapid renewal of endometrial glands, stroma and vasculature (29). During this proliferative stage, estrogen is the dominant hormone. As the levels of this hormone rise, estrogen receptors (ER) and progesterone receptors (PR) are induced in the epithelium and stroma and there is extensive proliferation of glandular epithelial cells in the functional layer (29). Angiogenesis occurs simultaneously to allow immune cell surveillance, oxygen supply to the new tissue and dispose waste (1,30). Angiogenesis is important during menstruation for repair of the vascular bed, during the rapid endometrial growth of the proliferative phase and during the secretory stage when spiral arterioles show significant growth and coiling (30). Numerous angiogenic factors have been identified in the human endometrium at the time of endometrial repair, though the vascular endothelial growth factor (VEGF) family genes is thought to be the most important (31). MMPs also play a key role in angiogenesis by degrading the extracellular matrix and permitting the migration and tube formation of endothelial cells (31).

After regeneration, the endometrium undergoes secretory changes mediated by the increasing levels of progesterone. The implantation window coincides with the circulating peak of progesterone levels and depends on endometrium modifications, which occur as a result of the prior exposure to estrogen following increased levels of progesterone. The key endometrial events associated with the implantation window are the increased expression of chemokines and cytokines, the onset of decidualization and the presence of increased numbers of leukocytes including uterine natural killer (uNK) cells (32).

1.2.1 Decidualization

In the mid secretory phase of the cycle, the elongated, fibroblast-like stromal cells of the proliferative tissue begin to differentiate into larger, round, secretory and epithelioid-like decidual cells. Decidualized stromal cells (DSC) are also characterized

by rounding of the nucleus, expansion of the rough endoplasmatic reticulum and Golgi complex, and cytoplasmatic accumulation of glycogen and lipid droplets (33). Characteristic secretory products include prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP1). Upon decidualization, these cells produce extracellular matrix (ECM) proteins such as type IV-collagen, laminin, decorin, fibronectin and heparan sulphate proteoglycans (34).

Decidualization of the endometrium occurs only in species in which placentation involves breaching of the luminal epithelium by the trophoblast (35). It has been implied that the decidual process is necessary to protect the integrity of the mother against inflammatory signals and oxidative stress (36).

In contrast to many species, human decidualization occurs independently of the presence of an implanting blastocyst and it affects all compartments, including junctional zone myocytes, spiral arteries, local immune cells, and the endometrial, epithelial and stromal compartments (33). It is a progressive process, initiated around the terminal spiral arteries of the superficial endometrial layer, which is also present in pregnancy and ultimately involves the entire endometrium (35).

Microarray analysis has been used to elucidate the extension and magnitude of the molecular mechanisms underlying the process of decidualization and potential paracrine interactions between DSCs and other cell types in the endometrium during normal cell cycles and in early pregnancy. On the basis of these studies, decidualization has been described as a process of sequential reprogramming of functionally related families of genes involved in cell cycle regulation, ECM organization, cell adhesion, cytoskeletal organization, angiogenesis, immune modulation of implantation, steroid hormone action and metabolism, stress response and apoptosis modulation (33,37,38).

Although the majority of original and review papers associate the initiation of the decidual process with the production of progesterone by the ovarian luteal cells, there are evidences that other physiologic effectors play a role in decidualization (39). *In vivo*, decidualization occurs about 10 days after the postovulatory increase of progesterone levels (40) and *in vitro* it takes 8 to 10 days for cells to decidualize under the stimulation of progesterone alone or in combination with estradiol (41). The initial and obligatory event that initiates the decidual process is the activation of the cyclic adenosine monophosphate (cAMP) pathway (33). cAMP is an ubiquitous second messenger molecule that is generated from adenosine triphosphate by adenylate cyclase. This enzyme is activated upon binding of a ligand to members of a family of G-protein-coupled receptors (GPCRs). These receptors mediate most of our physiological responses to hormones and neurotransmitters (42). Agonists activate

heterotrimeric (αβγ) G proteins by catalysing the replacement of GDP by GTP to the α-subunit resulting in the dissociation of α-GTP from βγ subunits (43). Then, α-GTP carries the signal to effectors, resulting in hormonal stimulation or inhibition of adenylyl cyclase by αs or αi respectively (43). This second messenger causes the activation of PKA, a cytoplasmic enzyme composed by two regulatory and two catalytic subunits (44). Upon binding of two cAMP molecules to each regulatory subunit, the latter undergo a conformational change, which results in the release and activation of the catalytic subunits. These may phosphorylate target molecules in the cytoplasm or diffuse into the nucleus modulating, in that way, the activity of transcription factors (45).

After ovulation, the endometrium is increasingly exposed to a variety of endocrine factors such as relaxin, prostaglandin E2 and gonadotropins, that are capable of stimulating cAMP production in stromal cells (39) by binding to GPCR coupled to the stimulatory Gsα protein (36). In fact, cAMP levels are higher in the secretory phase compared to the proliferative phase and it has been shown that treatment with a cAMP analog triggers the expression of the decidual markers, PRL and IGFBP1 (33). However, the decidual phenotype is not maintained in response to cAMP pathway alone. The inductive effect of cAMP on PRL production, by *in vitro* endometrial cells, was enhanced by medroxyprogesterone acetate (MPA), a metabolically stable progestin (39). Thus, cAMP signalling, via activation of the PKA pathway, sensitizes human endometrial stromal cells to progesterone (36). The mechanism by which cAMP potentiates PR activity remains a matter of debate, but it is thought to involve disruption of the interaction between the receptor and the corepressors NCoR and SMRT and to increase cooperation between coactivators such as SRC-1 and CBP (46). Besides this, it is also known that sustained elevated cAMP levels in human endometrial stromal cells induce the expression, or trigger the activation, of several downstream transcription factors, including forkhead box protein O1 (FOXO1), CCAAT/ enhancer-binding protein b (C/EBPb) and signal transducers and activators of transcription 5 (STAT5) (47). These decidua-specific transcription factors interact with PR, modulating the transcriptional competence of this nuclear receptor (48–50). Thus, decidualization requires a myriad of signalling pathways, transcription factors and cross talks to coordinate this temporary reprogramming of endometrial stroma (36).

At the time of implantation, decidua is responsible for sensoring embryo quality (51). Cyclic decidualization coupled to menstruation emerged as a strategy for early detection and active rejection of developmentally abnormal embryos that have breached the luminal epithelium. Low quality embryos elicit an endoplasmic stress response in human decidual cells compromising secretion of decidual factors, including

PRL and IGFBP1, essential for placental formation and fetal development (51). On the other hand, impaired cyclic decidualization of the endometrium facilitates implantation yet predisposes to subsequent pregnancy failure by disabling natural embryo selection and disrupting the maternal responses to embryonic signals (52). In fact, impaired decidualizing process is increasingly linked to a variety of pregnancy disorders, including infertility, recurrent miscarriages, utero-placental disorders, endometriosis, and endometrial cancer.

Decidua acquires the unique ability to control trophoblast invasion, to resist inflammatory and oxidative insults and to assuage maternal immune responses (Figure 2). The former is regulated through the creation of a dense cellular matrix, which promotes the trophoblast attachment and, at the same time, limits the aggressive invasion by the fetal tissue. This process relies on an intricate communication between trophoblast and maternal decidua, which requires proteolytic enzymes, such as MMPs and plasmin and their corresponding inhibitors (33,53).

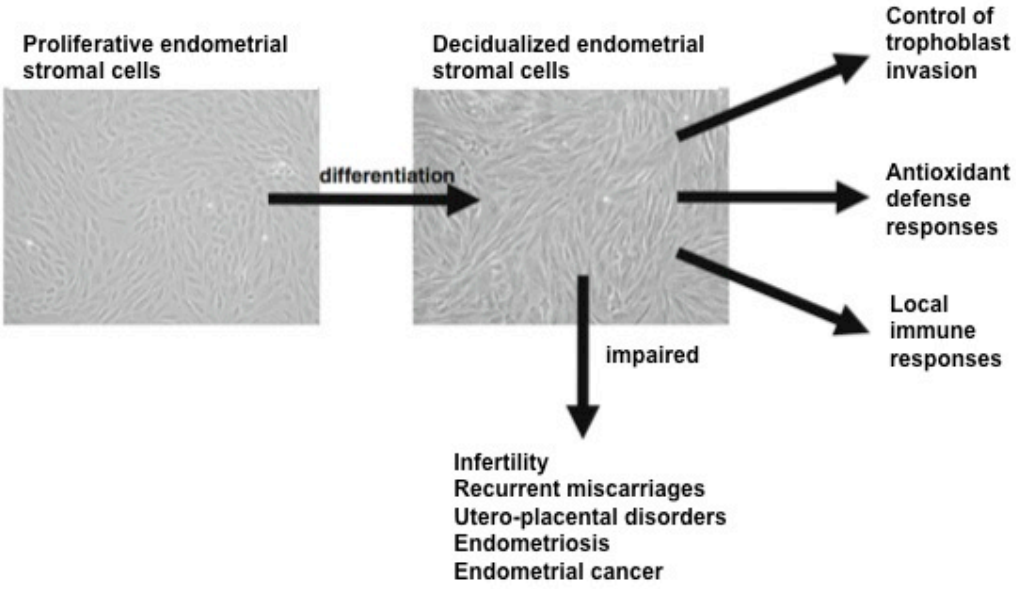

Figure 2. Decidual transformation of human endometrial stromal cells in vitro. Undifferentiated primary cells display a fibroblastic spindle-shaped morphology (left panel). After treatment with cAMP and progesterone cells acquire a decidualized epithelioid phenotype (right panel). This transformation in vivo underpins the acquisition of specialized functions. Impaired decidualizing process is increasingly linked to a variety of pregnancy disorders. (47)

In early pregnancy, maternal circulation to the placenta is extremely slow due to the plugging of spiral arteries by invading endovascular trophoblast. The human uteroplacental unit develops initially in a low-oxygen environment protecting the embryo against reactive oxygen species (ROS) (33,53). Unplugging of the arteries generates a burst of oxidative stress. Therefore, it is critical for the conceptus survival

to ensure the integrity of the feto-maternal interface. It is known that human endometrial stromal cells become extraordinarily resistant to oxidative stress-induced apoptosis upon decidualization (54) through the induction of various free radical scavengers, most notably superoxide dismutase 2, monoamine oxidases A and B, thioredoxin, glutaredoxin, and peroxiredoxin (47).

The human decidua also contains a high number of immune cells, such as macrophages, natural killer cells and regulatory T cells. This specific immune cells pattern represents a substantial entity, which exerts variable physiologic functions like maintaining immune tolerance toward implanted allogeneic embryo, surveillance against infections and neoplastic transformations, important functions related to placenta development (55). Deletion of these cells has deleterious effects on placental development, implantation, or decidual formation (56). On the other hand, upon decidualization, the differentiated stromal cells produce cytokines, chemokines and MMPs, that stimulate natural killer (NK) cells differentiation into uNK, monocytes recruitment and tissue remodelling (55). Therefore, a rigorous cross talk between decidual and immune cells is a requisite for pregnancy, though it is still a great challenge to understand the intricacy of fetal-maternal system behind a successful outcome.

2 Endocannabinoid system

Cannabis plant has been used for thousands of years as a source of useful fibre for textile manufacturing, a medicine and a recreational drug. The use of cannabis (or marijuana) as a psychoactive substance was introduced a long time ago in central Asia, spreading gradually to India, Minor Asia and Africa. It only reached Europe and America in the 19th century and now, it is one of the most consumed illicit drugs worldwide (57). The attractiveness of marijuana as a recreational drug is due to its ability to alter sensory perception and cause elation and euphoria (58).

Cannabis sativa contains over 60 cannabinoids as well as over 400 other chemicals (59). It was a major breakthrough in the 1960s, when Gaoni and Mechoulam identified delta-9-tetrahydrocannabinol (THC) as the most important psychoactive cannabinoid (60). Initially, it was suggested that due to its strong hydrophobic nature, THC would elicit its effects non-specifically by perturbing lipids. However, the synthesis and biological studies of enantiomers of THC and its synthetic analogues revealed that its principal pharmacological actions were enantioselective, a telltale sign of drug-

receptor interaction (58,61). Henceforth, the idea of a cannabinoid receptor and a whole endocannabinoid system was built and investigated.

Recently it was described that THC, by competing with the endogenous cannabinoids, may cause changes in the endocannabinoid signalling cascade with possible consequences for the biological functions of the endocannabinoid system (62) comprised by cannabinoid receptors, their endogenous ligands (endocannabinoids), enzymes for their synthesis and degradation and transporter (Figure 3).

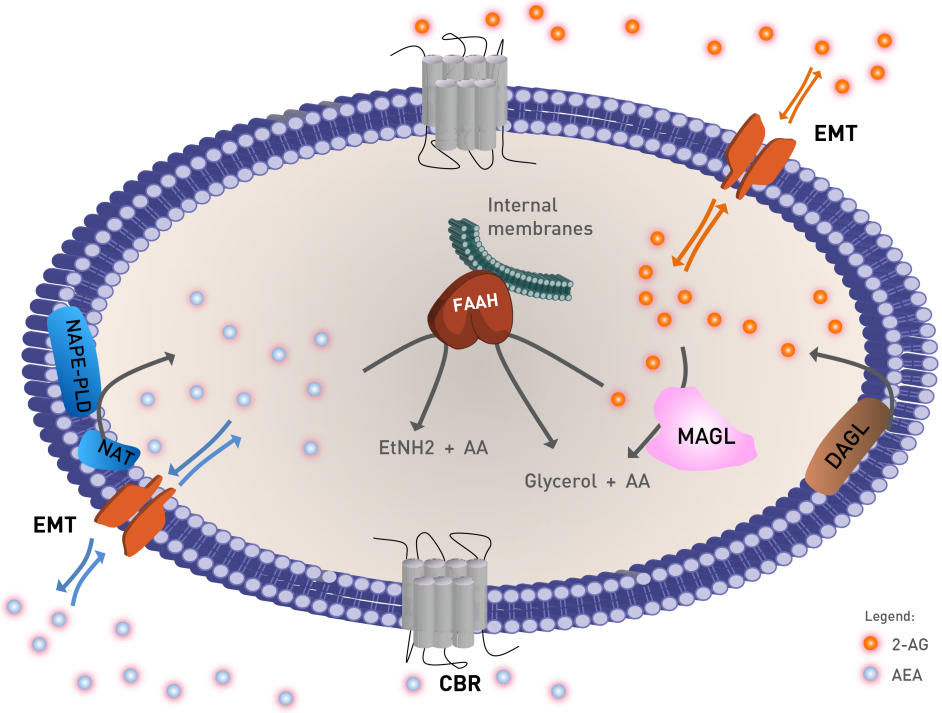

Figure 3. Components of the endocannabinoid system. Endocannabinoids, anandamide (AEA; blue circles) and 2-arachidonoylglycerol (2-AG; orange circles), are transported through cell membrane via the putative endocannabinoid membrane transporter (EMT). AEA is catalysed by the N-acyltransferase (NAT) followed by NAPE-specific phospholipase D (NAPE-PLD). 2-AG synthesis occurs through diacylglycerol lipase (DAGL). Endocannabinoids interact with cannabinoid receptors (CB). AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) into ethanolamine (EtNH2) and arachidonic acid (AA). 2-AG is hydrolyzed through monoacylglycerol lipase (MAGL) or FAAH into glycerol and arachidonic acid (66).

2.1 Receptors

Initially, it was found that the treatment of neuroblastoma cells with THC inhibited adenylate cyclase activity and that this action was stereoselective (63). This evidence allied to radioligand binding assays (64) and cloning of cDNA encoding a G-protein coupled receptor from rat cerebral cortex (65) were the starting point for the discovery of the first specific cannabinoid receptor (CB1). The existence of a second cannabinoid receptor, named CB2, was then identified. CB1 is the primary cannabinoid receptor in the central nervous system and is widely distributed throughout the brain

and at lower levels in peripheral tissues (immune system, vascular endothelium, intestine, liver, peripheral nerve synapses and reproductive system) (66). CB2 is expressed primarily by immune cells, including microglia (67). CB1 and CB2 receptors belong to the Class A (rhodopsin-like) of G-protein coupled receptors, which comprise seven transmembrane helices connected by three intracellular and three extracellular loops with a N-terminal extracellular domain and a C-terminal intracellular domain (68). Molecular modeling analysis based on the X-ray-crystal structure of similar receptors revealed the hypothesis that CB1 and CB2 present an opening between transmembane helices that allows ligand to pass from the lipid bilayer into the hydrophobic binding pocket (69,70). Pharmacological studies have shown that besides CB1 and CB2, endocannabinoids also interact directly with other molecular targets, including non-CB1, non-CB2 G-protein-coupled receptors and various ion channels. An orphan GPCR, GPR55, with the ability to interact with and be modulated by endogenous plant and synthetic cannabinoid ligands, was considered a novel cannabinoid receptor (71). GPR55 shares only 13.5% sequence homology with CB1 and 14.4% with CB2. The levels of GPR55 mRNA expression are notably between three- and tenfold lower than those reported for the cannabinoid CB1 receptor (72). GPR55 might also be activated by the bioactive lipid L-α-lysophosphatidylinsoitol and related lipids, which apparently do not bind to CB1 or CB2 (72). In addition to the receptors above mentioned, endocannabinoids were also described to interact with the orphan G-protein coupled receptor GPR119, with trasient receptor potential (TRP) channels (also known as vanilloid receptors) and peroxisome proliferator-activated receptors (PPARs) (66,73).

The discovery of the cannabinoid receptors was just the tip of the iceberg in the study of the endocannabinoid system. Today, the knowledge of this field of research encompasses the identification of endogenous ligands and their entire metabolism as well as the signalling pathways through which endocannabinoids exert their action.

2.2. Endogenous cannabinoids and their metabolism

Endocannabinoids are derivatives of integral components of cellular membranes and act as hydrophobic lipid messengers. These molecules have been described to be involved in basic biological processes, including cell choice between survival and death, immune response, neurotransmission, energy homeostasis and reproduction. These compounds exhibit “cannabimimetic activity”, that is, they act as “THC mimetics”.

The first endocannabinoid to be identified was arachidonoylethanolamide (AEA; anandamide) isolated from porcine brain (74), which was followed by the identification of 2-arachidonoylglycerol (2-AG). AEA and 2-AG are the best characterized members of the main families of eCBs, N-acylethanolamines (NAE) and monoacylglycerols (MAG), respectively (66). Although similar in structure and general function, these endocannabinoids exhibit large differences in terms of biochemical steps, receptor affinity, and breakdown pathways (75). Other endogenous ligands to cannabinoid receptors have been discovered: 2-arachidonoylglyceryl ether (noladin ether, 2-AGE), O-arachidonoylethanolamine (virodhamine), N-arachidonoyldopamine (NADA), N-arachidonoylglycine (NAGly) and Cis-9,10-octadecanoamide (oleamide or ODA) (66) (Figure 4).

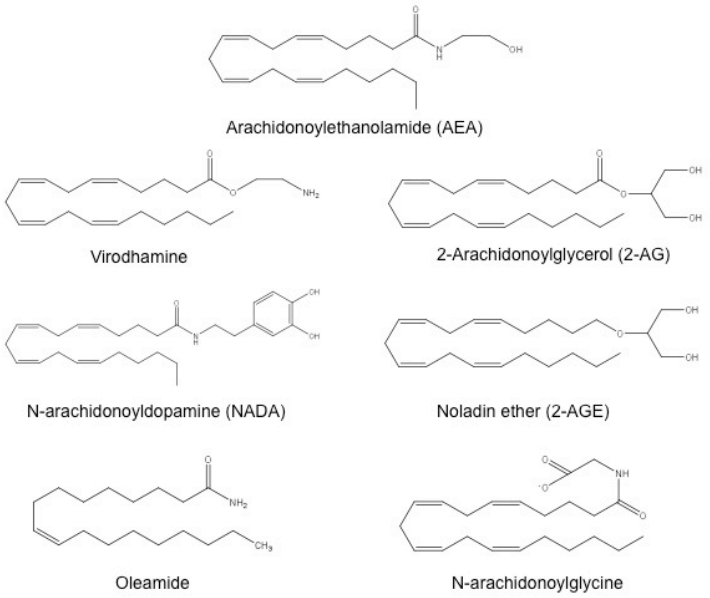

Figure 4. Chemical structure of endocannabinoids. Structure of the two main endocannabinoids (anandamide and 2-arachidonoylglycerol) and other endogenous cannabimimetic molecules that have thus far been identified (virodhamine, noladin ether, N-arachidonoyldopamine, N-arachidonoylglycine and oleamide).

The AEA precursor is N-arachidonoyl-phosphatidylethanolamine (NAPE), which is originated from the transfer of arachidonic acid from phosphatidylcholine to the head group of phosphatidylethanolamine, catalyzed by N-acyltransferase (NAT) (66,75). This occurs through enhanced intracellular Ca^{2+} concentrations, e.g. from cell depolarization or mobilization of intracellular Ca^{2+} stores (66). NAPE is then cleaved by a phospholipase D (NAPE-PLD) releasing AEA and phosphatidic acid (76). Recent studies have reported the existence of at least three other alternative pathways for

AEA synthesis (Figure 5). NAPE can be converted into phospho-AEA by a phopholipase C (PLC) and then dephosphorylated by a phosphatase. LysoNAPE, synthesised by a specific phospholipase A_2 (PLA_2), is hydrolyzed to AEA by a lysoPLD. NAPE can also be deacylated by ABHD4 generating glycero-p-AEA that is cleaved to AEA by a phosphodiesterase (75,77). Once taken up by the cells AEA is primarily degraded by fatty acid amide hydrolase (FAAH) releasing arachidonic acid and ethanolamine (66). Besides FAAH, AEA can be hydrolyzed by N-acylethanolamine-hydrolyzing acid amidase (NAAA) and oxygenated by COX-2, lipooxigenase isoenzymes (LOX), and by P-450 cytochrome (75).

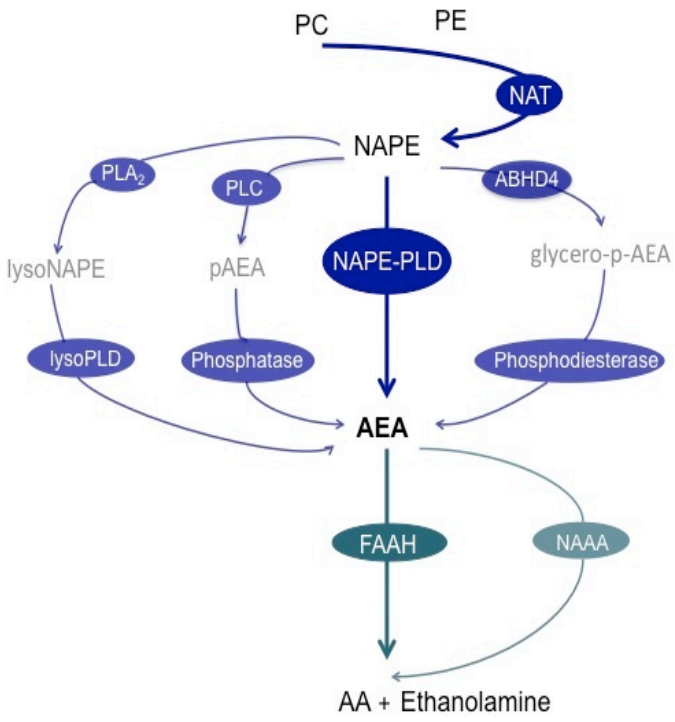

Figure 5. Metabolism of anandamide. AEA can be synthesized and degraded through multiple pathways. Transfer of arachidonic acid from 1-arachidonoyl-phosphatidylcholine (PC) to phosphatidylethanolamine (PE) is catalysed by NAT, thus generating the AEA precursor, N-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE can be directly cleaved by NAPE-PLD or through alternative pathways until AEA is synthetized. The major route for AEA degradation is the hydrolysis to arachidonic acid (AA) and ethanolamine.

After biosynthesis, anandamide is released to extracellular space to activate the cannabinoid receptors or act inside the cell in the TRVP1 or PPARs. Different hypothesis have been put forward to explain AEA transport, namely i) passive diffusion assisted by the formation of AEA-cholesterol complexes, ii) caveolae-related

endocytosis and iii) facilitated transport mediated by a selective and saturable transporter, the "anandamide membrane transporter" (AMT) (77).

Within the cell, AEA is either metabolized, which maintains the concentration gradient or is sequestred by lipid droplets (adiposomes) (78). FAAH is generally accepted as the key regulator for AEA gradient and consequent uptake. However there are experiments reporting AEA uptake in FAAH -/- mice, suggesting the existence of alternative AEA gradient regulators (78). An additional way of reducing the free intracellular AEA concentration is the binding to intracellular proteins. Fatty-acid-binding proteins (FABP)-5 and -7, albumin, heat shock protein 70 and the FAAH-like anandamide transporter protein (FLAT) have been identified as AEA intracellular binding proteins (AIBPs) (78). Although the majority of literature claims that AEA is synthesized and released exclusively "on demand", the existence of AIBPs and storage into adiposomes suggest that this idea should be reconsidered (77,79).

2.3 Signal transduction pathways

G-protein-coupled receptors can adopt multiple conformations, leading to different signalling events. These different conformations can be caused by different ligands, which can behave as agonist, antagonist or inverse agonist in another receptors. Inhibition of adenylyl cyclase via $G_{i/o}$ is the best characterized CB1 signalling pathway, as it results in reduced cAMP-stimulated PKA activity (80). A CB1-mediated activation of PKA has been shown to promote Ca^{2+} influx into neuroblastoma cells (81) and regulate K-type K^{+} channels in hippocampal neurons (82). Stimulation of cannabinoid receptors can also inhibit L-, N- and P/Q- type calcium channels (83).

G-protein coupled receptors can regulate cell proliferation, cell differentiation, cell motility and cell death through activation of MAPK pathways, which are organized hierarchically leading to activation of ERK1/2 (also known as p42/p44 MAPK), c-Jun N-terminal kinase (JNK), focal adhesion kinase (FAK), p38 MAPK or ERK5 proteins (66,83). These multiple pathways mediated by CB1 stimulation depend on the cell type and stimulus (80). CB1 activation by THC, anandamide or synthetic cannabinoids can also induce the activation of protein kinase B (PKB, also known as Akt) with the involvement of phosphoinositide 3'-kinase (PI3K) (84) (Figure 6). PKB plays a pivotal role in the regulation of basic cell functions such as energy metabolism and proliferation.

Cannabinoids can also modulate the intracellular levels of ceramide, which is an ubiquitous sphingolipid second messenger with an important role in the control of cell fate (85). Activation of CB1 can produce two peaks of ceramide. Short-term

ceramide generation involves sphingomyelin (SM) hydrolysis via sphingomyelinase (SMase) activation through the adaptor protein FAN and acts on metabolic regulation. Long-term ceramide generation may occur via serine palmitoyltransferase (SPT) induction and enhanced ceramide synthesis *de novo*, being linked to the induction of apoptosis (85).

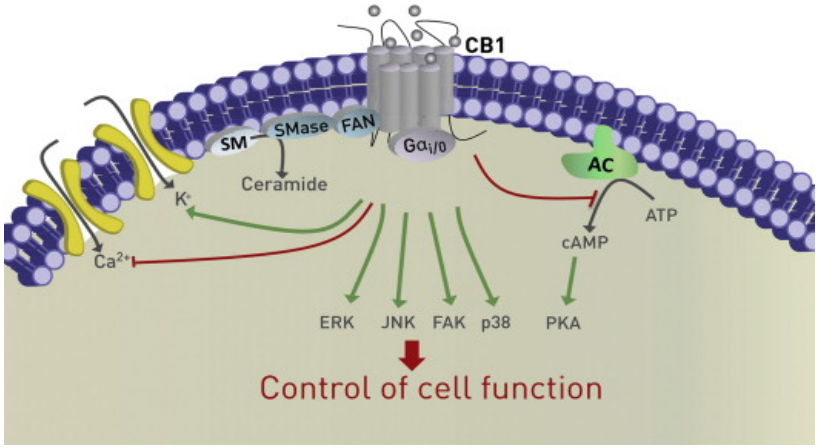

Figure 6. Cannabinoid receptor-coupled signalling pathways. Cannabinoids exert their effects through the stimulation of Gi/0 protein-coupled receptor signalling inhibition of adenylyl cyclase (AC). CB1 receptor mediates ceramide accumulation and modulates ion channels. Activation of the CB1 receptor can also stimulate various intracellular kinases, such as extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), focal adhesion kinase (FAK), and p38 MAPK (p38). All these events participate in the control of cell function by cannabinoids. (67)

2.3.1 Involvement of AEA in cell survival/death decision

The precise mechanisms through which cannabinoids induce apoptosis are still unclear, specially, because several mechanisms appear to be involved. Differences in cell type, receptor expression and/or the available concentrations of agonists result in activation of different signalling pathways. In fact, the proapoptotic activity of AEA in different cellular models has been shown to occur through the activation of different receptors (76).

In rat cortical astrocytes and human astrocytoma cells, proapoptotic signalling induced by AEA is mediated by CB1 receptor leading to activation of JNK and p38 MAPK (86). In the same cells, the activation of CB1 also leads to sphingomyelin breakdown through the adaptor protein FAN and independent of $G_{i/o}$ proteins, suggesting that a threshold might exist above which endocannabinoid-induced JNK and p38 MAPK activation would lead to cell death (86,87). It may be speculated that AEA binding to CB1 receptors modulates the balance among ERK, JNK and p38 MAPK, thus regulating the cell choice between proliferation and death (76). In rat

decidual cells, AEA induces cell death through CB1 (88) involving ceramide and p38 MAPK pathway (66). In addition it was also demonstrated that anandamide induced cell death in PC12 cells through activation of p38 MAPK and JNK proteins independent of CB1 or vanilloid receptors (89). Increasing data indicates that more sophisticated mechanisms should be involved in the regulation of cell death by cannabinoids. Potentially, AEA may exert its effects by direct inhibition of T-type calcium channels (90). Anandamide-induced cell death might also be mediated through membrane lipid rafts independent of cannabinoid or vanilloid receptors (89). Furthermore, recent findings enhance COX-2 contribution to cannabinoid-induced apoptosis. Treatment of human neuroglioma cells with the stable analogue of AEA (methanandamide) resulted in the induction of apoptosis via lipid raft-mediated events. The signal transduction pathway involved an increase of ceramide levels, expression of COX-2 and subsequent prostaglandin E2 synthesis involving p38 MAPK and ERK activation (91).

It has also been shown that cannabinoids are able to modulate, once more through CB1 receptors, the phosphatidylinositol 3-kinase/ protein kinase B (PI3K/PKB) pathway, which serves as a pivotal anti-apoptotic signal (92). This finding is of particular interest, because it points towards a protective role of cannabinoid receptors against programmed cell death (76). In human neuroblastoma CHP100 and lymphoma U937 cells, anandamide induced apoptotic bodies formation and DNA fragmentation, hallmark features of programmed cell death, via vanilloid receptors (93). In the same study, it was suggested that activation of cannabinoid receptors may also have a protective role against apoptosis. Thus, AEA might exert a pro-apoptotic activity by binding to vanilloid receptors and an anti-apoptotic action by binding to cannabinoid receptors (76). Protection against apoptosis following CB1 activation has been reported in several cellular models (76).

3 Endocannabinoid system in female fertility

Over the past half century, fertility rates have declined at an unprecedented speed, so that infertility has become an urgent public health issue specially in developed countries. Although, a great effort has been made to overcome infertility through assisted reproduction technologies (ART), it is still a major concern to understand the molecular mechanisms behind a successful pregnancy.

Reproductive functions are under a fine regulation exerted at multiple levels along the hypothalamic-pituitary-gonadal (HPG) axis. The formation of high quality gametes, followed by a successful pregnancy event, is the result of deep cell-to-cell communications (94). Numerous signalling molecules have been indentified as

potential modulators of reproductive activity. The endocannabinoid system (ECS) is one of these novel players implicated in the process of implantation and maintenance of pregnancy (95). Endocannabinoid signalling pathways are involved in fertilization, oviductal transport, implantation, embryo development and maintenance of early pregnancy (Table 1). AEA is now thought to be the key link between the developing embryo and the endometrium, ensuring synchronous development of the preimplantation embryo and the endometrium, thereby facilitating the embryo implantation during the 'implantation window' (96).

Table 1. Overview of the most important biological activities of endocannabinoids in the female reproductive organs.

Ovary	- oocyte maturation - folliculogenesis
Oviduct	- embryo transport
Endometrium	- embryo implantation - endometrial plasticity - endometrial cell motility - cytoskeleton rearrangement - endometrial cell proliferation - endometrial decidualization

3.1 Endocannabinoid levels during menstrual cycle

Endocannabinoids play an important role in folliculogenesis, ovulation, oocyte maturation and endometrium receptivity (97). Plasma anandamide levels fluctuate through the menstrual cycle with an increase at the follicular phase (1.45 ± 0.81 nM) compared with the luteal phase (0.77 ± 0.30 nM) (97). Higher levels occurring at the time of ovulation followed by a substantial decrease during the early to mid-secretory phase, when implantation occurs, are required to ensure a successful pregnancy (98).

Studies measuring FAAH expression in the peripheral lymphocyte during the secretory phase of the menstrual cycle around the time of implantation suggest that increased lymphocytic FAAH expression could be a controlling factor in plasma anandamide levels at this time (99). The highest FAAH activity and the lowest AEA concentration are registered on Day 21, a time point corresponding to the putative window of uterine receptivity for implantation (100). Besides, low FAAH and high AEA levels in blood were associated with spontaneous miscarriage (99). In addition, women undergoing successful ART show low levels of AEA at the time of implantation, compared with women who fail to become pregnant (98). The differential expression of

FAAH and NAPE-PLD in the endometrium, suggest a key role for these enzymes in controlling local AEA concentrations during the menstrual cycle (101).

The precise mechanisms by which endocannabinoids influence reproduction are uncertain, although the involvement of COX-2 may be one of them. Maintenance of appropriate AEA levels may be partly dependent upon oxidation by COX-2, which catalyzes the conversion of AEA to prostamides (prostaglandin-ethanolamides) (96). In fact, studies in mice showed that COX-2 might be essential for processes such as ovulation, fertilization, implantation and decidualization (102).

In addition to the direct effects proposed for endocannabinoids on reproduction, the ECS also interacts with sex steroid hormones and cytokines to regulate reproduction indirectly (96).

Estrogen response elements have been identified in FAAH gene sequence. Estrogen receptors down-regulate FAAH gene transcription, independent of its ligand and possibly through interference with the activity of positively regulating nuclear proteins such as AP-1, SP1, or other transcription factors (103). The overall relationship between estrogen and endocannabinoids can be described as "bidirectional" (94). In one direction, endocannabinoid activity reduces the release of estrogens via the central down-regulation of LH and GnRH (94,104). In contrast, decreasing FAAH activity and modulating CB expression, estrogen up-regulates AEA production (94).

Evidences show that endocannabinoids might also regulate negatively the release of progesterone by controlling LH release (94). On the other hand, progesterone up-regulates FAAH activity and thereby decreases AEA levels (96). In addition, cytokines such as LIF, Th1/Th2 and leptin that are essential for implantation, interact with the ECS, which ultimately have impact on fertility (96). Thus, for a successful implantation and maintenance of early pregnancy it is required a tight control of the hormone-cytokine network.

3.2 From the ovary to the uterus

Human oocytes express CB1 and CB2 receptors, whose localization changes with the different oocyte maturation stages. Immunostaining of normal human ovaries indicates that the ECS is present and widely expressed in the ovarian medulla and cortex, with more pronounced CB2 than CB1 immunoreactivity in the granulosa cells of primordial, primary, secondary, tertiary follicles, corpus luteum and corpus albicans (105). On the other hand, AEA metabolizing enzymes, FAAH and NAPE-PLD are only expressed in the granulosa and theca cells of secondary and tertiary follicles, in the

corpus luteum and in the corpus albicans (105). Thus, the ovary is able to produce AEA, which may play a role in folliculogenesis, preovulatory follicle maturation, oocyte maturity and ovulation. According to these results, the higher plasma AEA levels are registered at the time of ovulation, suggesting that the ECS may be required for the process of ovulation.

Embryo retention in the fallopian tube is one of the causes of ectopic pregnancy and consequent miscarriage. It has been shown that CB1 receptor regulates normal oviductal transport of embryos in mouse oviduct. In fact, genetic or pharmacologic silencing of cannabinoid receptor CB1 causes retention of a large number of embryos in the mouse oviduct, eventually leading to pregnancy failure (106). Also, wild-type females exposed to a stable AEA analogue (methanandamide) or natural THC had the same phenotype as $Faah^{-/-}$ mice with oviductal transport defects, which was rescued by CB1 antagonist treatment (106,107). A critical balance between AEA synthesis, by NAPE-PLD, and its degradation, by FAAH, both in mouse embryos and oviduct, creates a locally appropriate “anandamide tone” (107).

In the uterus, the endometrium represents a significant source of endogenous cannabinoids, and AEA levels are higher than in other reproductive tissues (108). Throughout the menstrual cycle, FAAH and NAPE-PLD expression in stromal and glandular compartments is responsible for the modulation of AEA concentrations as referred above.

Initially it was described that CB1 immunoreactivity is not modulated throughout the menstrual cycle (101). However, a study reported a dramatic increase in CB1 mRNA and protein in normal endometrial samples in the secretory phase likely due to progesterone modulation (109). On the other hand, CB2 expression levels are minimal at the end of the menstrual cycle and reach a peak during the late proliferative phase (101).

Recent data support the idea that the ECS plays an important role in the control of endometrial plasticity by regulating endometrial cell motility (108). For instance, methanandamide stimulates endometrial stromal cell migratory phenotype by inducing rapid changes in the cytoskeleton architecture and increasing electrical signal generated by K^+ channels (108). Cannabinoids may also interfere with the proliferation of endometrial cells. Treatment with the agonist WIN 55212-2 has an anti- proliferative effect on endometriotic and endometrial stromal cells mediated by a mechanism involving reduction of reactive oxygen species production and inactivation of the Akt pathway (110). Thus, the ECS may represent a promising approach in the treatment of deep infiltrating endometriosis.

3.3 Endocannabinoids at implantation and maternal-fetal interface

Attachment of the embryo to the luminal epithelium of the maternal uterus is a crucial step in mammalian reproduction. Synchronous development of the preimplantation embryo to the blastocyst stage and the uterus to a receptive stage is essential to the processes of implantation (Figure 7). On the maternal side it is critical the process of decidualization. It has been proposed that decidual cells are probably the main controllers of uterine endocannabinoid levels during rat pregnancy (111). The differentiation of stromal cells and apoptosis of the receptive decidua are crucial processes for the controlled invasion of trophoblasts in normal pregnancy. In rat, it was suggested that anandamide may play an important role in regulating apoptosis through CB1 and thereby modulate decidual stability and regression during pregnancy(88). This mechanism is triggered by ceramide synthesis *de novo* and p38 phosphorylation, followed by mitochondrial stress and ROS production (112). In human endometrial stromal cells, activation of CB1 by R(+)-WIN 55,212-2 mesylate inhibits decidualization process and promotes apoptosis of decidual cells by a cAMP-dependent mechanism (113).

Apart from the importance of the ECS in the modulation of decidualization, it has also been suggested that low levels of anandamide is important for implantation competence (114). Actually, the levels of uterine anandamide and blastocyst CB1 expression are regulated to synchronize preimplantation events and uterine receptivity for implantation in mice (114). Thus, encocannabinoid signalling is at least one of the pathways that determines the fate of implantation and ultimately successful pregnancy. However, the importance of the ECS does not end here. Evidence has been provided that it is also involved in early pregnancy events as well as the labor onset.

Placenta serves as an interface for the exchange of nutrients, gases, and wastes between the maternal and fetal compartments. Moreover, placenta can secrete many hormones and growth factors conducive to the success of pregnancy establishment and maintenance (94). It has been further demonstrated that CB1, FAAH, and NAPE-PLD are expressed in human placenta. The levels of FAAH in the human placenta increase towards the end of the first trimester of pregnancy, before declining by the early second trimester (115). Furthermore, findings suggest that the placenta may form a barrier preventing maternal-fetal transfer of anandamide and/or modulate local levels of this endocannabinoid by regulating FAAH expression throughout gestation (115).

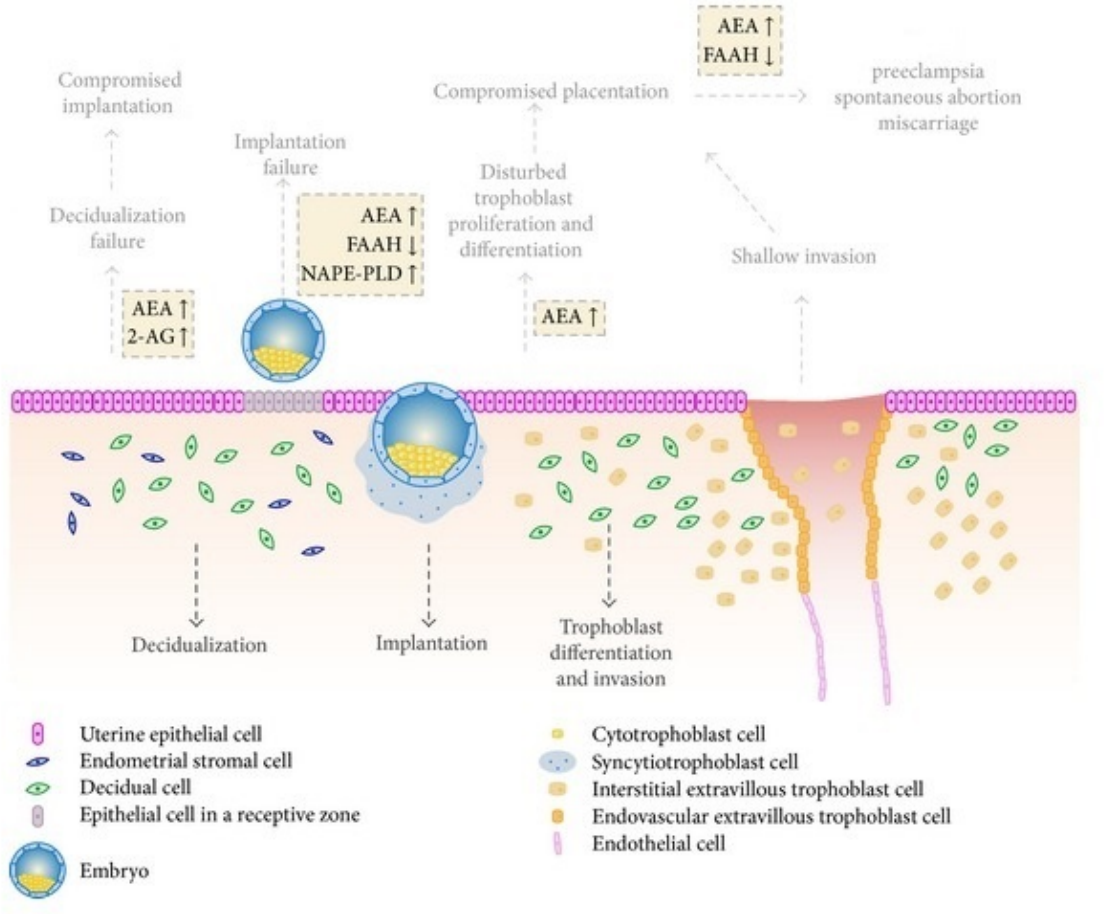

Figure 7. Fetomaternal interface and potential adverse effects of deregulated endocannabinoid levels. Based on rodents and human studies, endocannabinoids may be critically involved in decidualization, implantation, and trophoblast differentiation and invasion. Aberrant endocannabinoid levels (shown in yellow boxes) is reflected in compromised reprogramming of the endometrial stromal cells, implantation and placentation manifesting in ectopic pregnancy, intrauterine growth restriction, preeclampsia, miscarriage, and spontaneous abortion. Adapted from (116).

A recent study demonstrates that aberrant endocannabinoid signalling plays an important role in the pathophysiology of preeclampsia. The placental expression of NAPE-PLD is significantly higher in preeclamptic pregnancies, while FAAH exhibits an opposite result (117).

Additionally, during labour, AEA levels were 3.5-fold higher than non-labouring term levels (2.5 versus 0.7 nM), suggesting that increased levels of AEA are required to sustain normal labour, whereas successful implantation and pregnancy progression require low levels of this endocannabinoid (118).

4 Aims

Increasingly, the endocannabinoid system has been implied in a wide array of physiological and pathological processes. In female reproduction, recent evidence proposed that anandamide levels must be tightly modulated to assure a successful pregnancy. On the other hand, endometrium proliferation and differentiation are crucial processes for the creation of a favourable environment for receiving the blastocyst (implantation) and permitting embryo-fetal development. Over the last few years, a great effort has been made to understand the intricacies between the ECS and female fertility. Studies show that endocannabinoids might be related with endometrial stromal cells proliferation and also affect the process of decidualization, which could ultimately interfere with the development of a possible pregnancy. However, this relationship is still unclear and the mechanisms through which endocannabinoids exert their action on human endometrial stromal cells are unknown. Therefore, the aim of this work is to unveil the effect of anandamide in the stromal lining and to lift the tip of the veil to understand the underlying regulatory mechanism that might be behind this effect.

Since most studies that examine the role of endocannabinoids in the reproductive system have been carried out in rodents, it is still difficult to determine the effect of endocannabinoids in human female fertility. Besides, due to ethical reasons it is also difficult to use first trimester maternal tissues. In this study was used, for the first time, a telomerase-immortalized endometrial stromal cell line, St-T1b cell line. This cell line expresses the appropriate phenotypic endometrial stromal cell markers and the decidual response closely mimics that of primary cultures. Thus, we intend to investigate the effect of AEA either in non-differentiated as well as in the decidua formation. It will also be explored the optimum conditions for *in vitro* differentiation of human endometrial stromal cells. Additionally, the effect of anandamide will be evaluated in non-differentiated term decidua fibroblast cells, which has been described to resemble endometrial stromal cells.

II Methods

1 Materials

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), antibiotic–antimycotic (10,000 units/ml penicillin G sodium, 10,000 mg/ml streptomycin sulphate and 25 mg/ml amphotericin B) were supplied by Gibco Invitrogen Co. (Paisley, Scotland, UK). Insulin, 17-β-estradiol, 8-Br-cAMP, collagenase, 3-(4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium (MTT), Hoechst 33258, propidium iodide (PI), Triton X-100, Dnase-free Rnase A, staurosporine (STS), charcoal, isopropyl alcohol, chloroform, fluoroshield, Sigma Fast Red™ tablets and hematoxylin were from Sigma-Aldrich Co. (Saint Louis, MO, USA). AEA and AM 251 were supplied by Tocris Bioscience (Minneapolis, MN, USA). CytoTox 96 nonradioactive cytotoxicity assay kit was from Promega (Fitchburg, WI,USA). ^{3}H-thymidine was from Amersham Biosciences Corp. (Piscataway, NJ, USA). DPX and Aquamount medium was from VWR (Radnor, PA, USA). Giemsa was from Merck (Whitehouse Station, NJ, USA). Caspase-Glo® 3/7 luminometric assay was from Promega Corporation (Madison, WI, USA). VECTASTAIN ABC Kit was from Vector Labs (Burlingame, CA, USA). TRIsure was from Bioline (London, UK). KAPA SYBR FAST qPCR Kit was from Kapa Biosystems (Milmington, MA, USA). iScript cDNA Synthesis Kit was from BioRad (Hercules, CA, USA). Chemiluminescence detection kit was from Super Signal West Pico (Pierce, Rockford, USA). X-ray films were from Kodak XAR (Rochester, NY).

2 St-T1b cell line culture

The human endometrial stromal cell line St-T1b was kindly supplied by Dr. Birgit Gellersen from Endokrinologikum Hamburg, Hamburg, Germany. This cell line was obtained from uterine biopsy samples at the time of hysterectomy for benign gynaecological disorders and immortalized with human telomerase reverse transcriptase (hTERT) (119). These cells were maintained at 37ºC with 5% CO_2 in DMEM/F-12 medium supplemented with 10% charcoal-stripped FBS, 1 nM 17-β-estradiol (E_2), 1 µg/ml Insulin and 2% penicillin-streptomycin. FBS was previously inactivated for 1h at 56ºC and then treated with activated charcoal for 24 h. For each experiment cells were firstly incubated for 72 h in completed medium and then treated with only 1% FBS.

3 Decidualization of St-T1b cell line

St-T1b cells differentiation was performed in minimal medium 1 (MM1), which corresponds to DMEM/F-12 medium supplemented with 2% charcoal-stripped FBS and 2% penicillin/streptomycin. Decidualization was induced through three different treatments: 1) 0.5 mM 8-Br-cAMP and 1 µM MPA; 2) 20 nM forskolin (Fsk) and 1 µM MPA; 3) 10 µM PGE_2, 1 µM MPA and 10nM E_2.

4 Isolation and culture of term decidua fibroblast cells

The protocol for the isolation of cells from term decidua was implemented based on Richards et al, 1995 (120) with some additional steps. Term human placentas from caesarean section or vaginal delivery following uncomplicated pregnancies were obtained from Centro Materno-Infantil do Norte, Porto. All the procedures using term placentas were performed according to the Ethical committee of Centro Materno-Infantil do Norte. The decidua basalis was scraped from the chorionic membrane, dissected into small pieces and enzymatically digested in PBS with collagenase (1 mg/ml) for 1 h at 37 ºC with gentle shaking. The suspension was then centrifuged at 260 x g for 6 min and the pellet was ressuspended and incubated with ammonium chloride solution (0.8 g tissue culture grade NHCl, 0.084 g NaHCO and 0.037 g EDTA in 100 ml H_20) to lyse red blood cells during 5 min at 37 ºC. The resulting suspension was filtered through a 60 µm nylon mesh followed by a 40 µm nylon mesh and centrifuged at 260 x g for 10 min. The isolated cells were cultured in complete DMEM/F-12 medium. After 24 h, the non-adherent cells were removed and the cells were grown to confluence and sub-cultured. By subpassage 3, essentially all the cells were proliferating fibroblasts. Cells from 3 to 10 subpassages were used in these studies. To determine the ideal initial cellular density three different densities were tested (2 x 10^3; 2.5 x 10^3 and 5 x 10^3 cells/ per well) in 96-well plate.

5 Cell viability and cell proliferation

Cell viability was ascertained by the tetrazolium salt MTT assay and by measuring the LDH release. St-T1b cells and primary cells were cultured at a density of 2.5 x 10^3 cells/ well in 96-well plates and incubated with different concentrations of anandamide (0.01 µM – 50 µM) during 24, 48 and 72 h. After incubation, MTT (0.5 mg/ml) was added to each well and the plate was incubated for 2 h 30 min at 37ºC. The formazan was dissolved by the addition of DMSO: isopropanol mixture (3:1) and quantified spectrophometrically (540 nm). To verify the role of cannabinoid receptor, the antagonist of CB1, AM 251, was added 30 min prior to anandamide.

Lactate dehydrogenase is a stable cytosolic enzyme that is released from cells after cell lysis or membrane damage and thus it is extensively used as a marker for cytotoxicity. LDH release in the culture medium was measured using CytoTox 96 nonradioactive cytotoxicity assay kit according to the manufacturer's protocol. Absorbance was read in BioTek Power Wave XS.

To evaluate St-T1b cell proliferation after 48 h of treatment with anandamide, two tests were used: Sulphorhodamine B assay and Thymidine incorporation assay. Sulforhodamine B measures total protein content, while Thymidine incorporation assay measures the radioactive nucleoside, ^{3}H-thymidine, that is integrated into new DNA. For both assays, cells were plated in 96-well plates as for cell viability experiments.

In the sulphorhodamine B test, the medium was initially removed, cells were washed with PBS and fixed with trichloroacetic acid (4%) for 1 h on ice. After this period, plates were washed with PBS and air-dried at room temperature (RT). Sulpharhodamine B (0.4% (wt/vol) in 1% acetic acid) was added to each well and incubated for 30 min. After the incubation period, plates were quickly washed with 1% acetic acid, in order to remove unbound dye, and allowed to dry. Sulpharhodamine B was dissolved in tris-base (10 mM) and measured spectrophometrically (492 nm).

Thymidine incorporation was assessed by the addition of ^{3}H-thymidine (0.5 µCi). Three different times (8, 18 and 24 hours) of incubation with ^{3}H-thymidine were tested and 24 hours was selected. Thus, cells were treated for 48 h with AEA and in the last 24 h was added ^{3}H-thymidine. After a cycle of freezing/ defrosting, cellular content was harvested using a semi automated cell harvester (Skatrum Instruments, Norway) into a specific filter. After addition of scintillation cocktail, ^{3}H-thymidine incorporation was determined in a scintillation counter (LS 6500, Beckman Instruments, CA, USA). Results are expressed as relative percentage of the untreated control cells (100%).

6 Morphological studies

Morphological alterations induced by anandamide were evaluated by Giemsa and Hoechst staining. St-T1b cells and primary cells were plated at a density of 1.5 x 10^{4} cells/well in 24-well culture dishes and incubated for 48 h with different concentrations of AEA (1, 10 and 25 µM). After treatment, cells were fixed with methanol for 20 min on ice.

The Giemsa staining was used to analyse cell morphology. After fixation, cells were stained with Giemsa diluted in water (1:10) for 30 minutes, washed with water and

mounted in DPX. Cells were observed under the microscope Eclipse E400, Nikon equipped with image analysis software LeicaQwin. It was counted 2500 cells per slide in five different experiments.

To evaluate nuclear morphology, specifically chromatin condensation, cells were exposed to 0.5 mg/ml Hoechst 33258 in PBS for 20 minutes at room temperature, washed with PBS and mounted in fluoroshield. The cells were examined under a fluorescence microscope Nikon Eclipse Ci (Nikon, Tokyo, Japan) equipped with an excitation filter with maximum transmission at 360/400 nm and processed by Nikon NIS Elements v 4.0 software.

7 Immunocytochemistry

To determine the culture purity, vimentin, a fibroblastic cell marker, and cytokeratine, an epithelial cell marker, were localized by immunocytochemistry using an avidin–biotin alkaline phosphatase complex immunohistochemical technique - VECTASTAIN ABC Kit. Primary cells were plated at a density of 1.5 x 10^4 cells/well in 24-well culture dishes and incubated for three days. Medium was removed and cells were washed with PBS. Non-specific binding sites were blocked with diluted normal blocking serum for 20 minutes. Cells were incubated overnight with primary antibodies against vimentin (sc 6260; 1:500 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and cytokeratin (M 7018; 1:100 dilution) from Dako (Glostrup, Denmark) at 4ºC. After washing with PBS, cells were incubated with biotinylated secondary antibody for 30 minutes and then with Vectastain ABC-AP reagent. To initiate the reaction, alkaline phosphatase substrate solution Sigma Fast Red^{TM} tablets was added. The slides were counterstained with Mayer's hematoxylin solution and mounted in Aquamount. The choriocarcinoma cell line BeWo was used as a positive control for cytokeratine.

8 Cell cycle analysis

St-T1b cells were plated at a density of 17.5 x 10^4 cells in 25 cm^3 culture flasks and incubated with AEA (10 μM) during 48 h. Cells were tripsinized, centrifuged (260 x g, 5 min, 4ºC), resuspended in 0.5 ml PBS and fixed in 70% cold ethanol for at least 2 h. After fixation, cells were centrifuged and the pellet was washed three times with PBS. Cells were resuspended in 0.5 ml of DNA staining solution (5 mg/ml Propidium Iodide (PI), 0.1% Triton X-100 and 200 mg/ml Dnase-free Rnase A for 15 min at 37ºC. DNA content was analysed by flow cytometry based on the acquisition of 20 000 events (with a threshold of 500 000) in a BD Accuri™ C6 (Becton–Dickinson, San

Jose, CA, U.S.A) equipped with BD Accuri C6 software. Detectors for the three fluorescence channels (FL-1, FL-2 and FL-3) and for forward (FSC) and side (SSC) light scatter were set on a linear scale. Debris, cell doublets and aggregates were gated out using a two-parameter plot of FL-2-Area to FL-2-Width of PI fluorescence. Data was analysed using FlowJo Software (Tree Star, Ashland, OR, USA). Assays were performed in duplicate in three independent experiments.

9 Determination of caspase 3/7 activity

The Caspase-Glo® 3/7 was used to evaluate caspase 3/7 activities according to the manufacturer's protocol. St-T1b cells (2.5 x 10^3 cells/well) were incubated with anandamide (1 and 10 μM) for 36 h. As positive control, cells were incubated with Staurosporine (STS; 10 μM) for the last 3 hours of experiment. The resultant luminescence was measured in relative light units (RLU) using a 96-well Microplate Luminometer (BioTek Instruments, Winooski, VT, USA).

10 Western blot

To identify CB1 and CB2 proteins in St-t1b it was performed a western blot.

St-T1b cells were cultured at a density of 5 x 10^4 cells/well in 24-well culture dishes. After treatment, medium was removed and cells were washed with phosphate buffered saline (PBS). Lysis Buffer and protease inhibitor cocktail (1:100) were added to each well, and incubated for 15 min on ice. Cells were scraped and subjected to three cycles of freezing/defrosting. The protein fraction (supernant) was obtained after centrifugation at 14000 x *g* for 10 min and stored at -80 ºC. Total protein concentration was measured using the Bradford assay.

Protein samples (50 μg) were prepared in sample buffer, boiled for 3 min to denature proteins and subjected to 10% SDS-polyacrylamide and then transferred onto nitrocellulose membranes for 2 h 30 min. Membrane was incubated for 1 h in blocking solution (5% non-fat milk in PBS 0.1% Triton) at RT and then with the primary antibody solution overnight at 4ºC with agitation. Western blots were performed with antibodies against CB1 (1:100) and CB2 (1:100) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After washing, the membrane incubated with peroxidase-conjugated secondary antibody at RT for 1 h. Finally, blots were visualized by chemiluminescence detection kit and exposed to x-ray film. Membranes were stripped and reprobed with anti-β-tubulin antibody (1:500) to control loading variations. Brain and spleen tissue were used as a positive control for CB1 and CB2, respectively.

11 Quantitative Real-Time Polymerase Chain Reaction

To unfold the effect of anandamide during the process of decidualization, cells were co-incubated with 0.5 mM 8-Br-cAMP and 1μM MPA and anandamide (1 μM and 10 μM) for three days. The expression of the decidualization specific markers IGFBP1 was assessed by real-time polymerase chain reaction (real time-PCR). Analysis of cell transcript was carried out by total RNA extraction and respective cDNA synthesis followed by real time-PCR. Reactions were run in duplicate and normalized to the housekeeping gene, β2-microglobulin.

11.1 Isolation of total RNA from St-T1b cells

Total RNA was isolated from St-T1b cells by using TRIsure. Medium was removed and 500 μl of TRIsure per well was added to lyse the cells. To each sample was added 200 μl of chloroform and centrifuged at 12000 x g for 15 min at 4ºC. The aqueous phase was transferred carefully and RNA was precipitated with 250 μl of cold isopropyl alcohol after centrifuging at 12000 x g for 10 min at 4ºC. Supernatant was removed and the pellet was washed with 500 μl of 75% ethanol and centrifuged at 7500 x g for 5 min at 4 ºC. RNA was resuspended in Rnase free water and incubated for 10 min at 55 ºC to ensure it was completely solubilized.

RNA was quantified by Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and its purity was assessed by absorbance 260/280 and 260/230 ratios. RNA was stored at -80ºC until reverse transcriptase reaction.

11.2 Reverse transcriptase reaction: synthesis of cDNA

In order to synthesise cDNA from previously extracted RNA, the iScript cDNA Synthesis Kit was used according to the manufacturer instructions. Per reaction were used 4 μl of 5x iScript reaction mix, 1 μl of iScript reverse trancriptase, 1 μg of total RNA and Rnase free water until adjust the volume to 20 μl. Reagents were mixed carefully and centrifuged briefly. The samples were finally placed in the T100 Thermal Cycler (BioRad, Hercules, CA, USA) for the reverse transcriptase polymerase chain reaction (RT-PCR). DNA was stored at -20 ºC until Real time-PCR.

11.3 Real time-PCR

Real time-PCR of the previously synthesized cDNA was performed using the KAPA SYBR FAST qPCR Kit according to the manufacturer instructions. PCR mix was prepared in a 1.5 ml reaction tube by adding water, 1x KAPA SYBR FAST qPCR Master Mix (2x) Universal, 200 nM of each primer (Sigma-Aldrich Co., Saint Louis, MO,

USA), 1 µl of cDNA sample. Table 2 contains the information about primers sequence, accession number, annealing and melting temperature. Per reaction the final volume was set to 20 µl. The reaction plated was sealed, centrifuged briefly and placed in MJ Mini Thermal Cycler (BioRad, Hercules, CA, USA).

Table 2. Information about primer sequence, gene accession number and Real time PCR conditions for each target gene.

mRNA target	GenBAnk accession no.	Primes (5'-3')		Annealing temperature (ºC)	Melting temperature (ºC)
B2-microglobulin	NM 004048.2	Sense	TGCTGTCTCCATGTTTGATGTATCT	59	80
		Antisense	TCTCTGCTCCCCACCTCTAAGT		
IGFBP1	NM 000596	Sense	GAGATAACTGAGGAGGAG	59	77
		Antisense	CCAAAGGATGGAATGATC		

13 Statistical analysis

Statistical analysis was carried out by ANOVA, followed by the Bonferroni post hoc test to make pairwise comparisons of individual means (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA) when significance was indicated. The results are the mean of three independent experiments performed in triplicate. Data were expressed as mean±SEM and differences were considered to be statistically significant at $P < 0.05$.

III Results

1 Non-differentiated cells

1.1 Implementing cell line culture and further techniques

At the time this study started, the work with the St-T1b cell line had been recently initiated. Therefore, it was put a great effort to establish the conditions for the culture of these cells including the ideal cell density, FBS concentration and number of cell passages that could be used. Firstly, different concentrations of AEA were added to St-T1b cells cultured with 1 and 10% of FBS. With 10 % of FBS, AEA had no effect on these cells, so further studies were carried out using only 1% of FBS. It was also observed that after the nineth trypsinization, cells started to react differently to the treatment with endocannabinoids. Henceforth, St-T1b experiments were set from the second to the nineth trypsinization. In addition, different times of incubation were tested (8, 16 and 24 h) with ^{3}H-thymidine. Since the rate of proliferation was very low, it was chosen the 24 h for the thymidine incorporation assay.

As referred in the literature, to study the decidualization process the cells should be treated with MPA and cAMP. However, in our experiments the cells were consistently dying. So, as MPA was firstly dissolved in ethanol, it was tested MPA dissolved in water, but no differences were observed. Several concentrations of cAMP (0.1; 0.25 and 0.5 mM) with or without MPA were tried out, but cells continued to dye. It was then verified that this effect was immediate and so it was hypothesized that perhaps it was not the cAMP effects but its solvent. The medium pH was measured before and after treatment and different values were recorded, with an acidification of the medium after treatment. Although DMEM-F12 contains HEPES buffer, it was not enough to immediately stabilize the pH differences. In order to solve this, the medium with 0.5 mM cAMP and 1 µM MPA was prepared previously, stabilized in the incubator and only after that added to St-T1b cells.

This study also accompanied the establishment of primary cultures of term decidua fibroblasts. Different initial cell densities were tested, but it was concluded that the best option was to use the same as for St-T1b cell line.

1.2 Western blot analysis

The presence of the cannabinoid receptors, CB1 and CB2, in St-T1b cell line was evaluated by western blot (Figure 8). Results revealed that this cell line does express CB1. On the other hand, it was not detected the CB2 receptor protein, suggesting that St-T1b cell line might not express CB2.

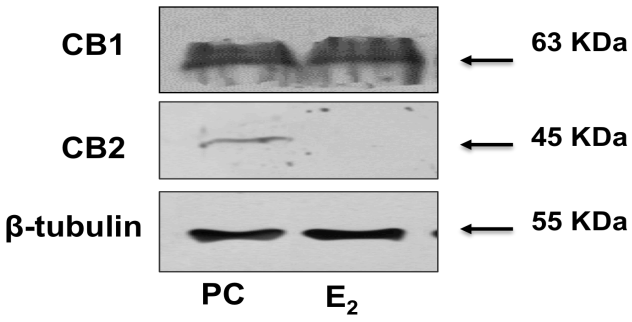

Figure 8. Cannabinoid receptors protein levels on St-T1b cell line. Representative western blots for CB1, CB2 and β-tubulin of protein extracts from St-T1b cells. (PC – positive control).

1.3 Cell viability

To unveil the effects of AEA in St-T1b cell line, cell viability and cytotoxicity were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and lactate dehydrogenase (LDH) release assays, respectively. AEA induced a reduction in cell viability in a dose-dependent manner between 10 and 50 μM.

The exposure to 5 μM of AEA had no effect on cell viability. However, at 24 and 48 h of treatment with 10 μM of AEA was observed a significant reduction of 19% in cell viability ($p < 0.0001$) (Figure 9A). After 24 h treatment with 25 and 50 μM, cell viability was reduced markedly to 50% and 30%, respectively.

A significant increase in LDH release was only observed for AEA concentrations higher than 25 μM (Figure 9B). Thus, MTT and LDH results showed that treatment with 10 μM of AEA leads to cell viability loss without evidences of cytotoxicity. Therefore, further experiments were performed with the concentration of 10 μM.

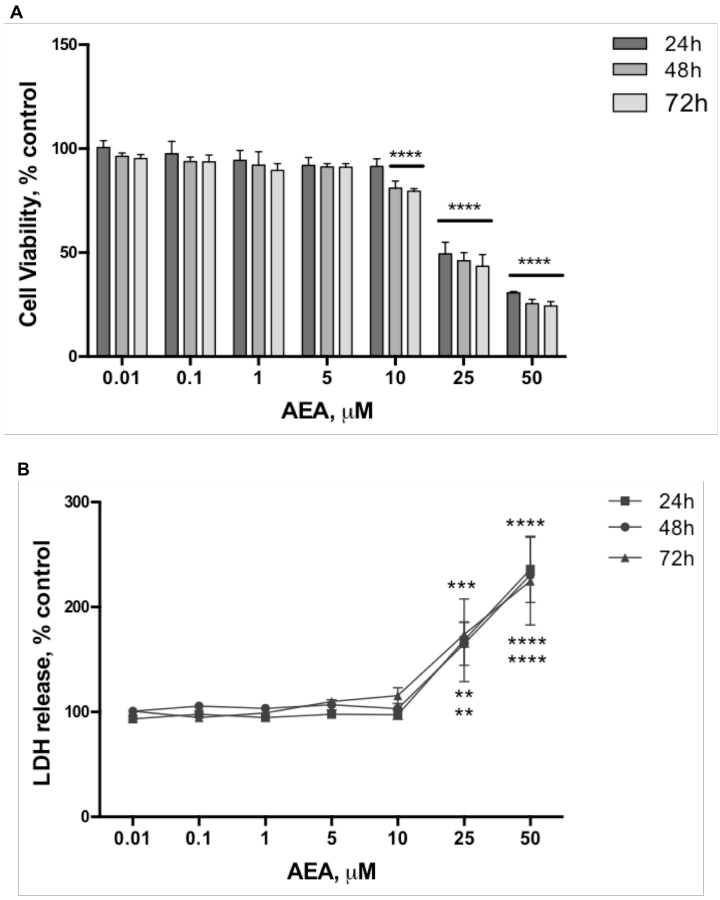

Figure 9. Effects of AEA on St-T1b cells viability. Cell viability of AEA-treated cells with different concentrations (0.01–50 μM) and at different times (24, 48, 72 h) accessed by MTT assay (A) and LDH release (B). Results are expressed as mean ± SEM of three independent experiments performed in triplicate. Significant differences between control and AEA-treated cells are denoted as **($p<0.01$); ***($p<0.001$) and ****($p<0.0001$).

To verify the involvement of the cannabinoid receptor CB1 in St-T1b cell death, CB1 was blocked with selective antagonist AM 251. After testing different concentrations of antagonist (0.1; 0.5 and 1 μM) the concentration used for the assays was of 0.1 μM. AM 251 did not prevent AEA-induced cell death (Figure 10).

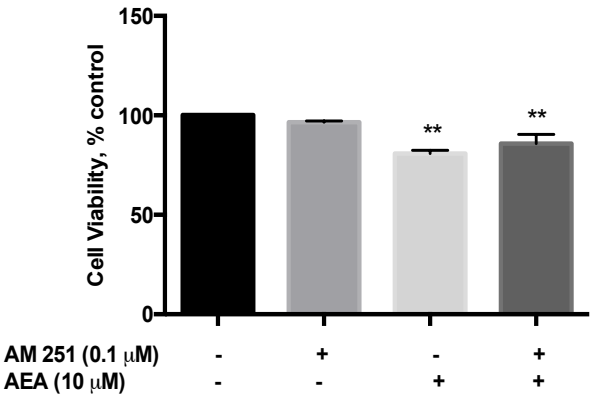

Figure 10. The effect of cannabinoid receptor in AEA-induced cell death of St-T1b cell line. Cells were treated with 0.1 μM AM 251, 10 μM AEA and antagonist plus anandamide. Results are expressed as mean ± SEM of three independent experiments performed in triplicate. Significant differences between control and AEA-treated cells are denoted as ** ($p<0.01$).

1.4 Cell proliferation

To analyse the effects on cell proliferation, sulphorhodamine B and thymidine incorporation assays were carried out. Sulphorhodamine B (Figure 11A) results showed that 1 μM of AEA had no effect on cell proliferation, whereas AEA at concentrations of 10 and 25 μM, induced about 22 and 50% decrease in cell proliferation, respectively. Besides sulphorhodamine B, it was also performed a thymidine incorporation assay (Figure 11B), which presented a much more dramatic effect of AEA in St-T1b cells proliferation. At 10 μM, AEA already decreased the rate of DNA synthesis to 53% and at 25 μM this effect was even more marked.

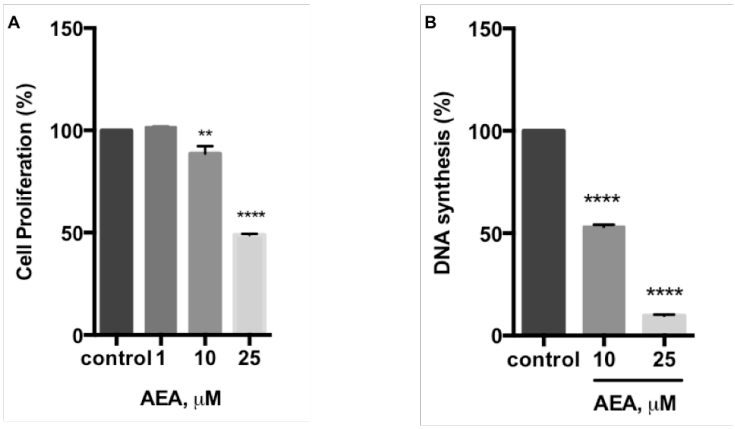

Figure 11. Effects of AEA on St-T1b proliferation assessed by sulphorhodamine B (A) and thymidine incorporation assay (B). Cell proliferation of St-T1b cells after 48 hours of treatment with different concentrations (1, 10 and 25 μM) of AEA. Results are expressed as mean ± SEM of three independent experiments performed in triplicate. Significant differences between control and AEA-treated cells are denoted as **($p<0.01$) and **** ($p<0.0001$).

1.5 Morphological studies

To investigate whether AEA treatment has an impact on St-T1b cell line morphology, cells were cultured for 48 h in the absence and presence of AEA (1 and 10 μM) and examined by Giemsa and Hoechst staining (Figure 12). The most marked differences were observed with 10 μM of AEA. Giemsa staining showed that under treatment, cells preserved general morphology, though binucleate cells were observed. A slight decrease in cell density was also detected. Hoechst staining indicated the presence of a slight increase in chromatin condensation, indicative of a possible mechanism of apoptosis. Binucleate cells presented smaller nuclei with chromatin condensation. Treatment with 1 μM of AEA showed, although sparsely, the presence of binucleate cells and chromatin condensation.

Through cell counting it was possible to confirm that after treatment with 10 μM, there was a significant increase of 10% of binucleate cells when compared to control (Figure 12G), while 1 μM of AEA did not lead to a significant increase in binucleate cells.

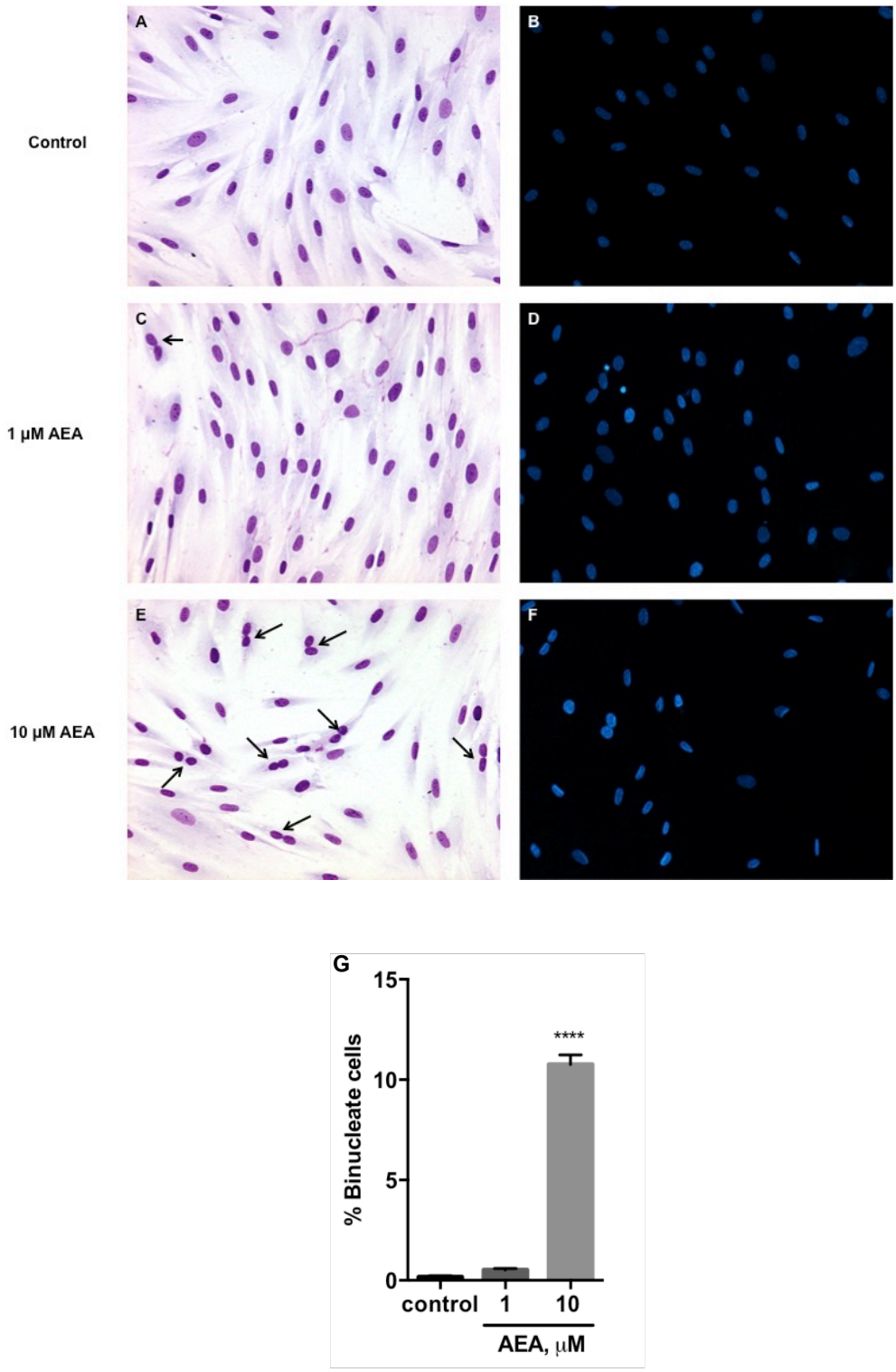

Figure 12. Effects of AEA on St-T1b cells morphology and binucleate cell counting. Giemsa staining (A, C and E) and Hoechst staining (B,D and F). Cells were incubated in the absence (A and B) and in the presence of 1 µM (C and D) or 10 µM (E and F) of anandamide for 48 hours. Arrows correspond to binucleate cells. (Original magnification, x200). G – Binucleate cell counting. Results are expressed as mean ± SEM of five independent experiments, where 2500 cells were counted per slide. Significant differences between control and AEA-treated cells are denoted as **** ($p<0.0001$).

1.6 Cell cycle analysis

To understand whether the reduction on cell viability and presence of binucleate cells was associated with an effect on cell cycle progression, DNA content was measured by flow cytometry (Table 3 and Figure 13). After 48 h of treatment, anandamide caused a significant cell cycle arrest in G_2/M (7.07 ± 0.49%, $p < 0.0001$) when comparing to control (2.1 ± 0.17%). On the other hand, it was observed a decrease in the percentage of cells in G_0/G_1. While in control 96.52 ± 0.33% of cells in G_0/G_1 was registered. After treatment this number was reduced to 89.7 ± 1.01% ($p < 0.0001$).

Table 3. Effect of anandamide in different phases of cell cycle progression in St-T1b cells treated during 48 hours.

Cell cycle	G_0/G_1	S	G_2/M
Control	96.52 ± 0.33	2.08 ± 0.35	2.1 ± 0.17
Treated	89.7 ± 1.01****	2.26 ± 0.19	7.07 ± 0.49****

Cells were treated with/without anandamide (10 μM). Treated cells were harvested, fixed and their DNA content was evaluated by PI labelling followed by flow cytometry analysis. Data are presented as single cell events in G_0/G_1, S and the G_2/M phases of the cell cycle. Results are expressed as mean ± SEM of three independent experiments performed in duplicate. Significant differences between control and AEA-treated cells are denoted as **** ($p<0.0001$).

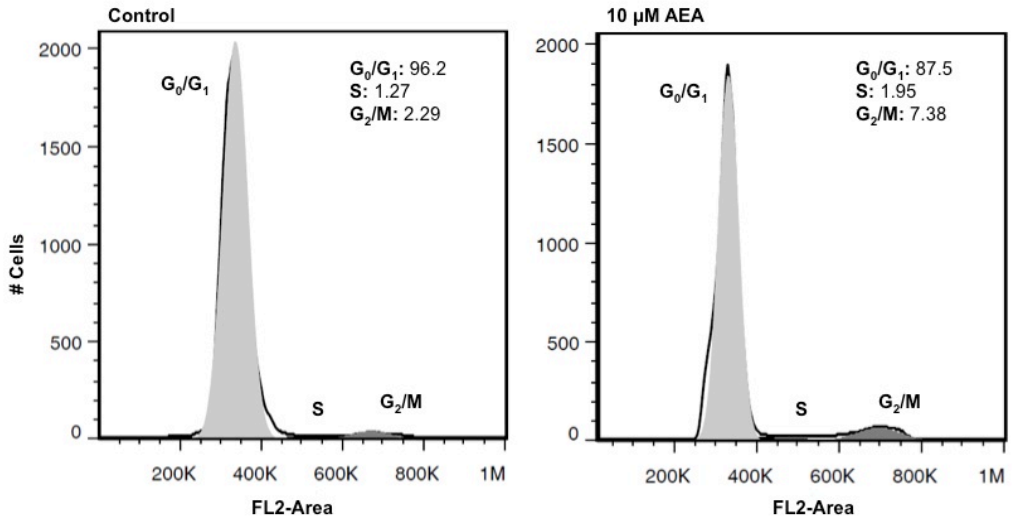

Figure 13. Representative histograms of cell cycle distribution of St-T1b cells control and treated with anandamide (10 μM) for 48 h. Histograms were obtained with FlowJo Software (Tree Star, Inc) by the application of the Watson mathematical model and are representative of one independent assay. The numbers indicate the percentage of cells in each cell cycle phase.

1.7 Caspase 3/7 activity

As described above, treated cells with AEA (10 μM) presented chromatin condensation in Hoechst staining, a feature of apoptosis. In order to confirm the occurrence of apoptosis, it was evaluated the activity of caspase 3/7 by luminescence (Figure 14). Treatment with AEA (10 μM) lead to an increase of 17% in caspase 3/7 activity in comparison to the control cells ($p < 0.5$). The addition of AM 251 did not reverse the AEA-induced caspase 3/7 activity. STS was used as a positive control for apoptosis.

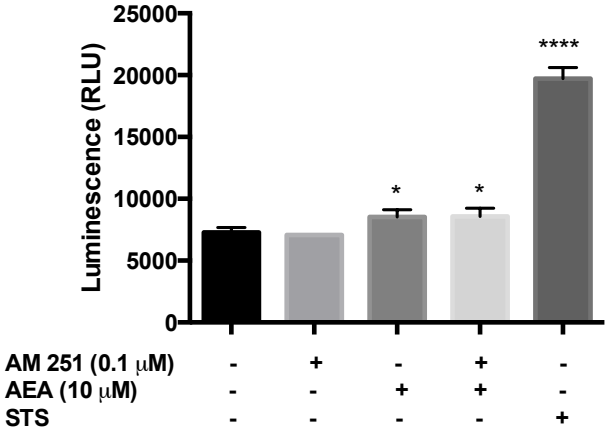

Figure 14. Caspase 3/7 activity of control and AEA-treated cells. Evaluation of caspase-3/7 activity in St-T1b cells after 36 h of culture with AEA (10 μM) and AM 251. STS (10 μM) was a positive control. Results are expressed as mean ± SEM of three independent experiments performed in triplicate. Significant differences between control and AEA-treated cells are denoted as * ($p<0.05$) and **** ($p<0.0001$).

1.8 Term decidua fibroblast cells viability

To ascertain the purity of the primary culture, expression of the cytoskeletal proteins vimentin and cytokeratin was analysed by immunocytochemistry (Figure 15). Cell population from primary culture of term decidua fibroblast cells were positive for vimentin and did not express cytokeratin, an epithelial cell marker. A choriocarcinoma cell line, BeWo cell line, was used as a positive control for cytokeratin.

To evaluate cell viability, MTT and LDH assays were performed as shown previously for St-T1b cell line (Figure 16). AEA induced a reduction in cell viability in a dose- and time-dependent manner. A significant loss in cell viability to 83.6% was observed for the concentration of 10 μM and after 48 hours of treatment ($p < 0.001$). Between 10 and 25 μM this effect was drastic with a decrease in cell viability to 26% at 24 hours of treatment with 25 μM. LDH release was similar to the observed for the St-

T1b cell line. A cytotoxic effect was observed after 24 h of treatment with 25 μM of AEA.

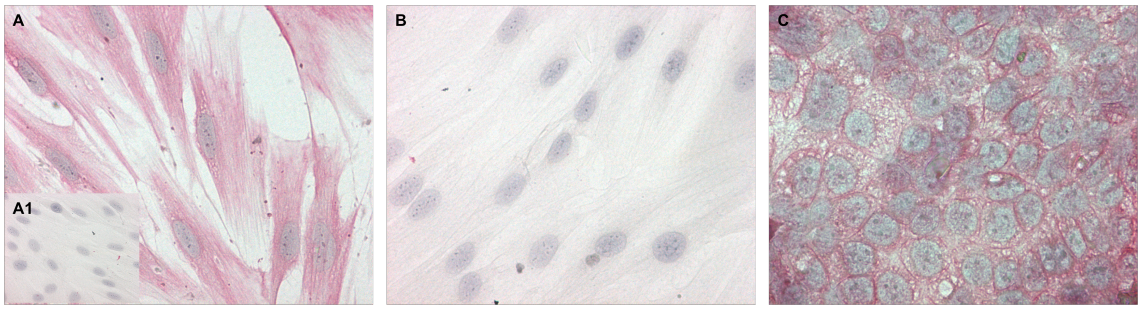

Figure 15. Detection of cytoskeletal proteins by immunocytochemistry. A - Term decidua fibroblast cells were positive for vimentin and negative (B) for cytokeratine. C - The choriocarcinoma BeWo cell line was used as a positive control for cytokeratine. (A1 - negative control; original magnification, x400).

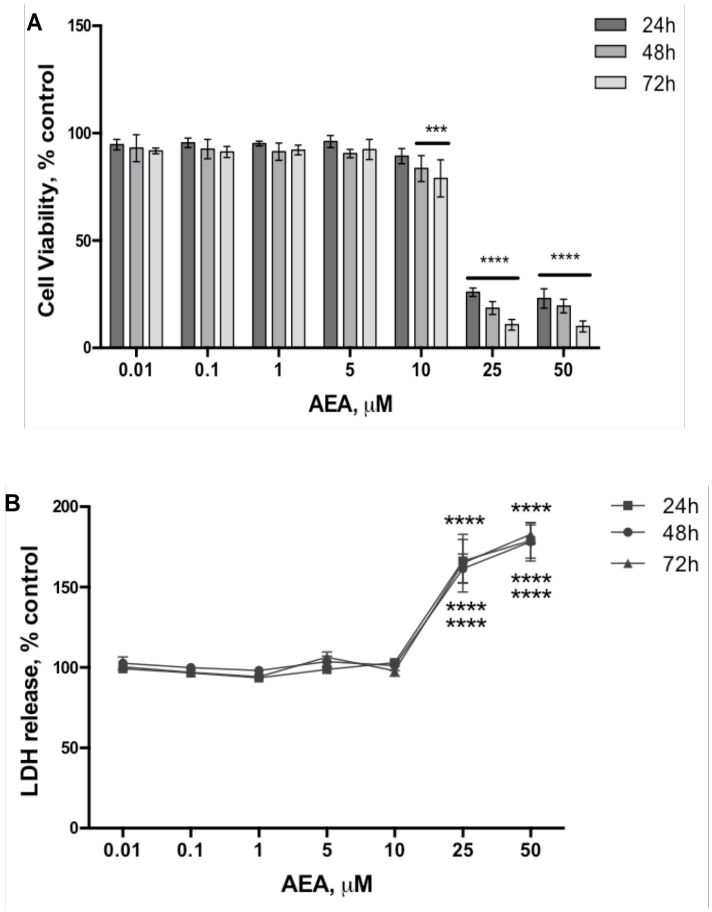

Figure 16. Effects of AEA on term decidua fibroblast cells (A and B) viability estimated by MTT assay and LDH release. Cell viability of AEA-treated cells with different concentrations (0.01–50 μM) and at different times (24, 48, 72 h) assessed by MTT assay (A) and LDH release (B). Results are expressed as mean ± SEM of three independent experiments performed in triplicate. Significant differences between control and AEA-treated cells are denoted as ***($p<0.001$) and **** ($p<0.0001$).

1.9 Term decidua fibroblast cells morphology

The effect of AEA on term decidua fibroblast cells morphology was determined by Giemsa and Hoechst staining (Figure 17). Cells were treated with 1 and 10 μM of AEA for 48 hours and compared to control. In the control condition, cells presented a confluence of about 90% with an elongated morphology. It was possible to observe a scarce number of binucleate cells. The treatment with 1 μM of AEA showed a very similar morphology to the control. It was not denoted a decrease in cell number and there was still some dispersed binucleate cells. Treatment with 10 μM of AEA led to marked alterations. It was detected a decrease in cell density and a larger quantity of binucleate cells, although general morphology was not affected.

Similar to St-T1b cell line, hoechst staining showed an increase of chromatin condensation after treatment with AEA. At 1 μM of AEA this effect was still not marked.

Although the presence of binucleate cells was already detected in the control, AEA-treated cells (10 μM) showed an increase of 6% (Figure 17G).

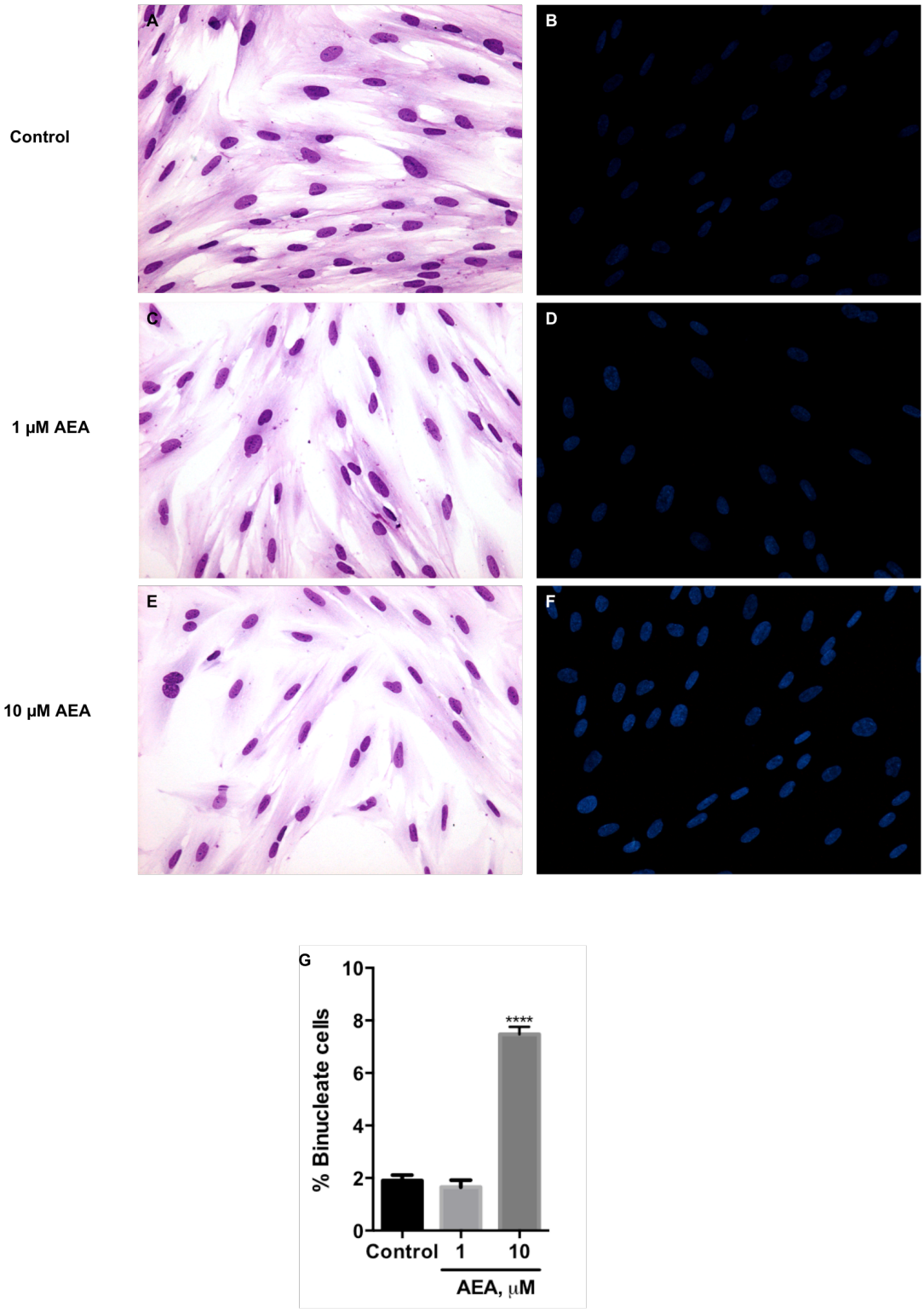

Figure 17. Effects of AEA on term decidua fibroblast cells morphology and binucleate cell counting. Giemsa staining (A, C and E) and Hoechst staining (B,D and F). Cells were incubated in the absence (A and B) and in the presence of 1 µM (C and D) or 10 µM (E and F) of anandamide for 48 hours. Arrows correspond to binucleate cells. (Original magnification, x200). G - Binucleate cell counting. Results are expressed as mean ± SEM of five independent experiments, where 2500 cells were counted per slide. Significant differences between control and AEA-treated cells are denoted as **** ($p<0.0001$).

2 Differentiated cells

2.1 Efficiency of cAMP, forskolin or prostaglandin E2 in decidualization

When compared to non-decidualized St-T1b cells, each of the experimental conditions showed morphological differences (Figure 18). After treatment with cAMP, cells became larger and rounder, typical characteristics of decidualized cells. Through Giemsa staining it was possible to observe that nuclei acquired a rounder morphology. Cells under treatment with forskolin also showed morphological differences, though cells were still slightly more elongated than with cAMP. Although prostaglandins and estradiol induced to some extent a round cellular shape, it was the treatment that most resembled the non-decidualized control.

The various differentiation treatments presented a reduced cell number, when compared to the non-decidualized situation. However, between cAMP, forskolin and PGE_2 + E_2 it was possible to denote differences, where cAMP had a lower number of cells than prostaglandins and estradiol conditions.

IGFBP1 mRNA levels were also evaluated as a biochemical marker of decidualization (Figure 19). While PGE_2 + E_2 showed similar levels to the non-decidualized control, forskolin induced an increase in IGFBP1 though these were not considered statistically significant. On the other hand, cAMP was the only differentiation factor to induce a statistically significant increase in IGFBP1 mRNA levels. Thus, these results indicate that cAMP + MPA was the most efficient treatment to induce decidualization of St-T1b and was henceforth used in the following experiments.

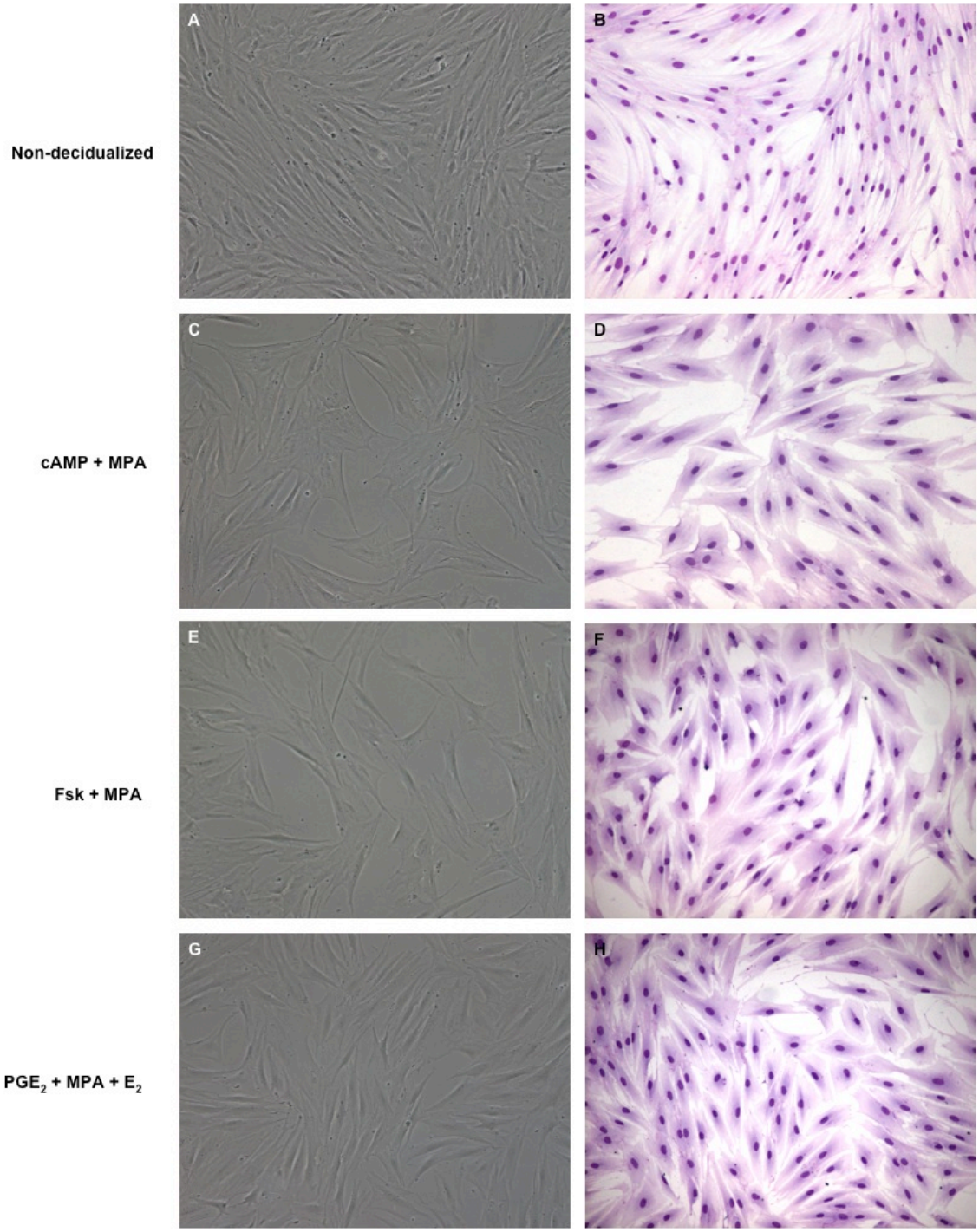

Figure 18. Effects of various differentiation molecules on St-T1b cells morphology. Phase contrast microscopy (A, C, E and G) and Giemsa staining (B, D, F and H). Non-differentiated cells (A and B) compared with cAMP + MPA (C and D); Forskolin + MPA (E and F) and PGE_2 + MPA + E_2 (G and H). (Original magnification, x100)

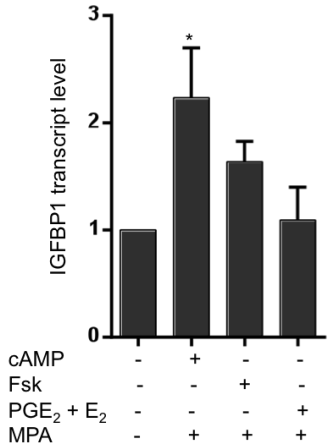

Figure 19. Transcript levels of IGFBP1 in St-T1b cells treatment with various differentiation molecules. cAMP was the most efficient treatment. All numerical data are expressed as mean ± SEM against control and differences were considered to be statistically significant when $P<0.05$.

2.2 Effect of AEA on decidualization

Although these experiments are still not concluded, preliminary results indicate that AEA caused an effect in the differentiation of St-t1b cell as assessed by the expression of the decidual marker IGFBP1 (Figure 20). AEA at a concentration of 10 μM induced a decrease in IGFBP1 mRNA when compared to the cells cultured in the absence of AEA, while 1 μM AEA had no negative impact on decidualization.

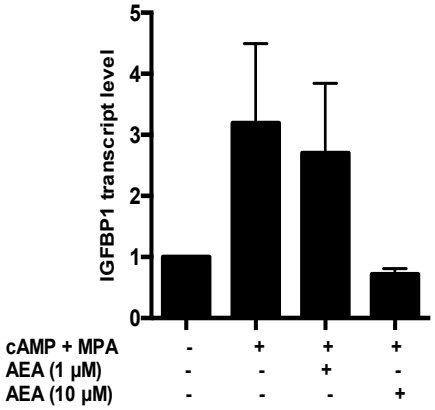

Figure 20. Preliminary results for the effect of AEA on St-T1b cell line differentiation.

IV Discussion

The uterus undergoes cyclic morphological and physiological changes with the sole purpose of creating a suitable environment for implantation of the blastocyst. Under the influence of ovarian steroids, the endometrium is subjected to the processes of proliferation and differentiation culminating in specific period of receptivity. In addition, endocannabinoids are new lipid mediators involved in reproduction namely, in folliculogenesis, embryo transport through the oviduct and the establishment of a receptive endometrium. Thus, the aim of this work was to clarify what is the impact of AEA on human endometrial stromal cells and whether a deregulation of the endocannabinoid system might affect the proliferation and differentiation of these cells.

Our results showed that AEA induced a reduction in cell viability, which might be elicited by an anti-proliferative effect and/or the induction of apoptosis. To test the effect on St-T1b cells proliferation, it was used the sulforhodamine B and the thymidine incorporation assays. Sulforhodamine B measures total protein content and does not distinguish between viable and dead cells (121), while thymidine incorporation assay measures directly DNA synthesis. Although both techniques have been broadly used to study proliferation during the last few years, the thymidine incorporation assay is more accurate. The results obtained by these two methods showed undoubtedly that anandamide has an anti-proliferative effect on endometrial stromal cell line, which is in accordance to other studies that reported AEA as an anti-proliferative agent in different cell types. On cholangiocarcinoma cell lines Mz-ChA-1, HuCCT-1, CCLP-1 and SG231, anandamide exert an anti-proliferative effect by activation of GPR55 (122), while in neuroblastoma cells this effect was achieved through a lipid raft-dependent mechanism (123).

Based in this anti-proliferative effect there is an emergent research area that studies the potential benefit of designing novel anti-cancerous therapies in association with endocannabinoids. In fact, the modulation of endocannabinoids has been implied as a resource for novel therapies in steroid hormone-dependent cancers, such as breast, prostate and endometrial cancer (124). This anti-proliferative effect has also been reported as a promising approach in the treatment of endometriosis (110), a common disease characterized by the presence of endometrial tissue outside of the uterine cavity.

In addition, the decrease in cell proliferation was not the only action exerted by AEA on St-T1b cells. Hoechst staining revealed that this endocannabinoid could also induce chromatin condensation, a feature of apoptotic process. There are two main

apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Both pathways end at the point of the execution phase, when effector caspases 3/7 are activated. In turn, effector caspases activate cytoplasmic endonuclease, which degrade nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins (125). To confirm the occurrence of apoptosis it was evaluated the activity of caspases 3/7 after 36 hours of treatment with AEA. It was observed an increase of 17% when compared to control, confirming the induction of apoptosis.

Apoptosis has been identified at three different phases of the menstrual cycle, namely the early proliferative, late secretory, and menstrual phases (126). Hence, apoptotic cells were not detected during the late proliferative phase until the midsecretory phase. At the beginning of the late secretory phase, apoptosis in the stromal cells spreads gradually to almost all components of the functional layer (126). Thus, this programmed cell death might be an important mechanism in the regulation of the menstrual cycle in women. Since anandamide levels are higher in proliferative phase than in secretory phase (127), this endocannabinoid might be related with the control of apoptosis throughout the menstrual cycle. On the other hand, the decreased endocannabinoid tone during the “window of implantation” is attributed to the maximal expression of degradation enzymes in the secretory phase (128). Thus, a disruption of the ECS with an alteration of synthesis/degradation pathways, may affect adversely the endometrial stromal layer. This could have a negative impact for the success of implantation, since it would alter the uterine environment.

In addition, it was also demonstrated that the reduction in cell viability is independent of cannabinoid receptor, CB1, which is in accordance with some studies already published. Maccarrone et al., described that AEA induces apoptosis in human neuroblastoma CHP100 and lymphoma U937 cells via vanilloid receptors and assigned a protective role to cannabinoid receptors (93). On the other hand, membrane lipid rafts have been implicated in AEA-induced cell death, in a mechanism independent of cannabinoid or vanilloid receptors (89).

Surprisingly, by light microscopy it was observed the presence of binucleated cells after AEA treatment, corresponding to 10 % of total population. Since, AEA had an antiproliferative effect and simultaneously led to the presence of binucleate cells it was investigated what was the real impact of this endocannabinoid in the cell cycle regulation. Thus, by flow cytometry, it was shown that AEA promoted a significant cell cycle arrest in G_2/M phase. Compared to control, the cell population at the G_2/M phase after treatment was approximately 3 times fold, being accompanied by a decrease in G_0/G_1 phase. These results revealed that the anti-proliferative effects of anandamide

are essentially due to the retention in G_2/M, which prevents cells to enter in the G_1 phase. Several natural and synthetic (endo)cannabinoids have been described to inhibit cell proliferation, arrest the cell cycle and induce cell death, mainly through apoptosis and autophagy (129). In gastric cancer cells, treatment with AEA led to retention in G_2/M phase and a subsequent increase in apoptosis (130). The same result was observed in breast cancer cells after treatment with THC (131).

Cytokinesis is a highly ordered process, requiring an intricate interplay between cytoskeletal, chromosomal, and cell cycle regulatory pathways (132). To determine whether AEA-induced binucleated cells formation was due to inhibition of cytokinesis, it would be important to monitor the re-organization of microfilaments and microtubules proteins, actin and tubulin, respectively. Also, one of the central regulators of cytokinesis is the small GTPase RhoA and it has been extensively described that defects in Rho signalling is one of the mechanisms that results in cytokinesis failure (132,133).

Cytokinesis arrest and consequent binucleate cells have been associated with DNA damage and programmed cell death (134). By contrast, differentiation of hepatocyte progenitor cell line, Lig-8, into mature hepatocytes is defined by binucleation (135). Following this line of thought, it has been suggested that AEA, in rat adipocytes, regulates cell differentiation (136). AEA increased adipocyte differentiation in primary cell cultures in a concentration- and time- dependent manner and induced *PPARγ2,* CB1, FAAH, and *COX-2* expression (136). In fact, the presence of binucleate cells has been reported in human decidualization (39), though their importance has never been addressed. In rodents, stromal cell polyploidy during decidualization limits the life span of decidual cells for the accommodation of the growing embryo (137). On the other hand, polyploidization is a feature of terminal differentiation (decidualization) and though it can be induced by multiple pathways, like apoptosis, immune system, mitochondrial function and tumors, the polyploidization of stromal cells is an elaborate regulatory system (138). For instance, in mice, the DEDD protein is involved in the induction of apoptosis and at the same it has been implicated in decidual cell polyploidization, being highly expressed at G2/M phase (138). The low levels of AEA at the time of implantation are important for the endometrium receptivity, implying that the presence of binucleate cells prior to the time of decidualization might jeopardize this process.

In addition, primary cell culture had results consistent with St-T1b cell line. AEA induced a loss of cell viability and led to a significant increase of binucleate cells. However, contrary to St-T1b cell line, term decidua fibroblast cells also presented binucleate cells in the absence of AEA. After mid-pregnancy despite high levels of

circulating progesterone the decidua begins a slow process of regression, which continues to term. In rat, this regression is characterized by an increase of proteins that promote apoptosis such as p27, Bax and caspase-3 (139). Thus, term placenta was exposed to the processes of differentiation, proliferation, regression and apoptosis and additionally to anandamide levels throughout pregnancy, which might explain the presence of these binucleate cells.

In this work we were also interested to understand the AEA effect during the differentiation of stromal to decidual cells, a process designated decidualization. First were tested multiple inducing treatments. cAMP was the most efficient as revealed by the alterations in morphology and expression of IGFBP1, a biochemical decidual marker, followed by forskolin and PGE_2 morphological and biochemical markers followed by forskolin and PGE_2. Although PGE_2 is known to induce differentiation of primary cultures (140), the St-T1b cells revealed a weak degree of differentiation. Forskolin, which is an agent able to enhance cAMP levels (141), showed marked morphological changes accompanied by an increase in the expression of biochemical markers. These results confirmed that St-T1b differentiation could respond to different conditions, though with different intensities.

Preliminary experiments indicated that AEA might inhibit the process of decidualization as shown by the decrease of the expression of IGFBP1, a decidual marker. Kessler et al, associated this effect to the activation of CB1 and stimulation of apoptosis by a cAMP dependent mechanism (113).

In summary, the work developed in the scope of this thesis may serve as basis for understanding the effect of AEA on human endometrial stromal cells. It was established that this endocannabinoid is responsible for an anti-proliferative and apoptotic outcome. It leads also to a significant increase of binucleate cells. Nevertheless, it is still required to investigate the mechanism behind the AEA- induced apoptosis and the presence of binucleate cells. Since the process of decidualization is a prerequisite for a successful pregnancy and the majority of this work was performed in non-differentiated cells, it is imperative to continue and reproduce these experiments in the differentiation process. This would enable to explore if differentiated cells enhance the protection of stromal layer against anandamide deregulation.

Furthermore, these results indicate that a tight AEA levels modulation is crucial for women cyclic endometrial preparation for pregnancy. However, a bigger issue is imposed when we consider that recent studies demonstrate that THC and other exogenous cannabinoids exert potent effects on the ECS homeostasis. Cannabis is one of the most consumed illicit drugs worldwide and a peak of drug usage is

registered among 18 and 25 years old women. This fact is of great concern, since it might be behind for one of the reasons of idiopathic infertility, which is diagnosed in 15% to 30% of couples. If cannabis use alters the ECS, including metabolism enzymes, then, it will ultimately interfere with endocannabinoids levels and possibly with female fertility.

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